α4 Integrin Enhances Susceptibility of B Cells to Epstein-Barr Virus Infection

Dissertation

zur

Erlangung der naturwissenschaftlichen Doktorwürde

(Dr. sc. nat.)

vorgelegt der

Mathematisch-naturwissenschaftlichen Fakultät

der

Universität Zürich

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Zürich, 2014

The experimental work presented in this thesis was performed at the Division of Infectious Diseases at the Children's University Hospital Zurich. This thesis was supervised by Prof. Dr. David Nadal (Children's University Hospital Zurich), Prof. Dr. Ueli Grossniklaus (Institute of Plant Biology, University of Zurich), Prof. Dr. Christian Münz (Institute of Experimental Immunology, University of Zurich), Prof. Dr. Roberto F. Speck (University Hospital Zurich), Prof. Dr. Alexandra Trkola (Institute of Medical Virology, University of Zurich) and Dr. Michele Bernasconi (Children's University Hospital Zurich).

I hereby declare that I have composed this thesis independently and only used the resources stated herein.

Zurich, February 2014

Patricia Krukowski

Acknowledgements

A special thanks goes to my supervisor Prof. Dr. David Nadal for his advice, encouragement and contribution to this work.
I also thank Dr. Michele Bernasconi fort his guidance through the project and valuable discussions about my research.

Special thanks also go to the entire Infectiology group who directly or indirectly contributed to the success of this work. Maria Gierisch and Patricia Jaaks for their encouragement and friendship.

Thanks to my good friends Simone Peter, Isabelle Stöckli, Anita Rösch, Jasmin Grählert and Wilma Felix for their encouragement, support and valuable relationships.

I would like to dedicate this thesis to my family for their unflagging love and support throughout my life; this thesis would have been impossible without them. My mother and father, Teresa and Janusz Krukowski and sister Esther Krukowski for their care, financial support and love.
Mario Wickert for encouraging me to pursue my dreams and for his love and support.

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Summary

The γ-herpesvirus Epstein Barr virus (EBV) has evolved during millions of years and has adapted very well to its human host. More than 95% of adults worldwide are infected with EBV and carry it lifelong in the B cell pool.

EBV can transform B cells *in vitro* and was shown to be associated with several human cancers like B cell lymphomas and nasopharyngeal carcinoma.

EBV entry into B cells requires the binding of viral glycoprotein gp350/220 to the cell surface receptor CD21 followed by the interaction of the viral tripeptide complex gHgLgp42 with HLA class II to initiate the fusion of the viral envelope with the host cell membrane. EBV entry into B cells is known to occur without the expression of CD21, but little is known about the contribution of other cellular receptors for viral attachment. Nevertheless, integrins are considered to be likely involved in CD21independent EBV entry into B cells. Integrins belong to a large family of transmembrane cell surface receptors that mediate adhesion between cells and their extracellular environment. We previously showed that expression of β1 integrin increases the susceptibility of memory B cells, originating from tonsils, to EBV infection. However, other integrins might be involved as well, since a whole set of integrins are expressed on B cells.

In the present work we show that integrins indeed play a role in EBV entry into B cells. We characterized Burkitt lymphoma (BL) cell lines and primary B cells from tonsils (TBCs) for their integrin expression and found that the BL cell line Raji expresses α4β1 integrin more abundantly than TBCs and that Raji cells are more susceptible to EBV infection than TBCs. Overexpression of the subunit α4 integrin facilitated EBV entry into the BL cell line Akata31. Our data indicate that α4β1 integrin plays a role in the process of B cell infection by EBV and contributes to enhanced susceptibility of B cell subsets. Furthermore, to investigate the function of α4 integrin in depth, we established the best technique for gene knockdown in the BL cell line Raji cells. However, silencing with specific siRNA against α4 integrin was not efficient.

It will be important to verify these findings on primary B cells. For this reason we established the conditions for efficient separation of naïve and memory B cells from tonsils by magnetic-activated cell sorting (MACS).

Zusammenfassung

Der γ-Herpesvirus Epstein-Barr- Virus (EBV) hat sich während Millionen von Jahren entwickelt und hat sich sehr gut an seinen menschlichen Wirt angepasst. Mehr als 95 % der Erwachsenen weltweit sind mit EBV infiziert und tragen es lebenslang in ihren B-Zellen.

EBV kann B-Zellen *in vitro* transformieren und es wurde gezeigt, dass EBV mit mehreren menschlichen Krebsarten wie beispielsweise B-Zell-Lymphomen und Nasen-Rachen-Karzinoma assoziiert ist. Der Eintritt von EBV in die B-Zellen erfordert die Bindung des viralen Glykoproteins gp350/220 an den auf der Zelloberfläche befindlichen CD21-Rezeptor, gefolgt von der Interaktion des viralen Tripeptid gHgLgp42 Komplexes mit HLA-Klasse II-Oberflächenmolekülen, um eine Fusion der Virushülle mit der Wirtszellmembran zu initiieren. Es ist bekannt, dass der Eintritt von EBV in B-Zellen ohne die Expression von CD21 geschehen kann, aber über die Rolle anderer zellulären Rezeptoren für die virale Anhaftung ist wenig bekannt, dennoch ist es wahrscheinlich, dass Integrine beteiligt sind. Integrine gehören zu einer grossen Familie von Transmembranrezeptoren, welche die Adhäsion zwischen Zellen und ihrer extrazellulären Umgebung vermitteln.

Wir haben bereits gezeigt, dass die Expression von Integrin β1 die Anfälligkeit von Gedächtnis-B-Zellen aus Tonsillen gegenüber einer EBV-Infektion erhöht. Andere Integrine könnten auch beteiligt sein, da eine ganze Reihe von Integrinen auf B-Zellen exprimiert werden.

In der vorliegenden Arbeit zeigen wir, dass Integrine tatsächlich eine Rolle bei dem EBV Eintritt in B-Zellen spielen. Wir charakterisierten Burkitt-Lymphom (BL) Zelllinien und primäre B-Zellen aus Tonsillen (TBCs) bezüglich der Expression von Integrinen und haben gefunden, dass die BL-Zelllinie Raji α4β1 Integrin höher exprimiert als TBCs und Raji-Zellen sind für EBV-Infektion anfälliger als TBCs. Die Überexpression der Untereinheit α4-Integrin erleichtert EBV den Eintritt in die BL -Zelllinie Akata31. Unsere Daten zeigen, dass α4β1 Integrin eine Rolle im Prozess der B-Zellinfektion durch EBV spielt und zu einer erhöhten Empfindlichkeit von B-Zell-Untergruppen beitragen kann. Um die Funktion der α4-Integrine zu analysieren, haben wir das Gen-Silencing von α4-Integrin in den BL-Zelllinie Raji optimiert, jedoch konnte kein effizienter Knockdown erreicht werden. Es wird wichtig sein, die gesamten neuen Erkenntnisse in primären B-Zellen zu überprüfen.

Daher schufen wir die Voraussetzungen für eine effiziente Trennung von naiven und Gedächtnis-B- Zellen aus Tonsillen durch magnetische Zellsortierung (MACS)

Introduction

Epstein-Barr virus (EBV)

Epstein-Barr virus (EBV) is a member of the human γ-herpesvirus family. EBV has coevolved with human beings during million of years and, consequently, the virus has become well adapted to its human host. More than 95% of the human population is infected with EBV and carries it lifelong in the B cell pool with very little threat to the healthy host. A primary infection in early childhood is generally asymptomatic and is transmitted via saliva among family members (51). An infection occurring during late childhood or adolescence may manifest symptomatically and is termed infectious mononucleosis (IM) (51, 157) Additionally, IM has been associated with an elevated risk of Hodgkin lymphoma (HL), implicating a role for Epstein-Barr virus (EBV) in HL development (65). During the latent viral infection phase, virus production or periodic reactivation is continued at a low level to avoid immune recognition and, conclusively, EBV may spread through kissing. Therefore, IM is also termed *kissing disease*.

EBV was not identified because of an acute illness, but was discovered within cells derived from a Burkitt's lymphoma (BL) by Epstein and his colleague Barr in 1964 (44). The virus is now estimated to be present in 96% of endemic BL and also in a variety of other tumors (36). In B lymphocytes and EBV-associated malignancies different viral transcription patterns are observed and have been referred to as latency types 0, I, II and III.

Table 1. EBV latency programs and EBV-associated malignancies.

Latency program	EBNA-1	LMP-1 LMP-2A LMP-2B	EBNA-2 EBNA3s/ EBNA-LP	EBERs BARTs	Associated malignancies
0				+	
I	+			+	Burkitt's lymphoma, Gastric carcinoma
II	+	+		+	Hodgkin's lymphoma, Nasopharyngeal carcinoma, T cell lymphoma
III	+	+	+	+	Lymphoproliferative disease, Infectious mononucleosis

Adapted from Masucci, 2004 (112).

EBV has the ability to transform resting B cells into latently infected lymphoblastoid cell lines (LCLs). In EBV transformed LCLs, a small subset of viral gene products, the so-called latent proteins, are constitutively expressed: six EBV nuclear antigens (EBNA): EBNA-1, EBNA-2, EBNA-3A, EBNA-3B, EBNA-3C and EBNA leader protein (LP); and three latent membrane proteins (LMP): LMP1, LMP-2A and LMP-2B. In addition LCLs also show expression of the small, non-polyadenylated (non-coding) RNAs EBER-1 and EBER-2 and transcripts from the BamHI A region (BARTs) (136). This expression pattern of all latent gene products is referred to as latency III and is associated with autonomous B cell proliferation.

Studies with recombinant EBV forms, lacking individual latent genes, confirmed the absolute requirement for EBNA-2, LMP-1 in the transformation process and have highlighted a crucial role for EBNA-1, EBNA-LP, EBNA-3A and EBNA-3C (189).

Malignancies associated with EBV infections

Denis Burkitt first described Burkitt's lymphoma (BL) in 1958 in Uganda (22). He observed rapidly enlarging tumors, which were found to be dependent on the climatic and geographical conditions of equatorial Africa. This lead to the suggestion that a vector-borne virus might be responsible for the development of these tumors (21). In 1964, EBV was first discovered by Epstein, Anchong and Barr: They identified herpes virus-like particles in cells cultured from a BL biopsy by electron microscopy (44).

In the late 1960s, it was found that sera from BL patient had higher antibody titers to EBV antigens than to sera from healthy control donors (62). Only a few years later, the ability of EBV to efficiently transform resting B cells *in vitro* and to induce tumors in non-human primates was confirmed (64, 114, 130). At the same time, it was found that EBV was also present in undifferentiated nasopharyngeal-associated carcinoma (NPC) (63, 193). Later studies showed an association between EBV infection and a variety of other human tumors including B cell malignancies such as Hodgkin's lymphoma (HL) (179) and lymphoproliferative diseases arising in immune-suppressed patients (166), certain types of T cell lymphomas, and epithelial tumors such as gastric cancer (33). All these tumors are characterized by the presence of multiple extrachromosomal copies of the circular viral genome in the tumor cells and expression of the EBV-encoded latent genes, which appear to contribute to the malignant phenotype (135).

EBV-associated Burkitt's lymphoma

Three clinical variants of BL are described according to their geographic distribution: endemic, sporadic, and immunodeficiency-associated (68). The endemic BL (eBL) characteristically occurs in Africa and affects children at the age of four to seven years, with a male to female ratio of 2:1. Affected are the bone of the jaw and other facial bones, as well as kidneys, gastrointestinal tract, ovaries, breasts, and other extranodal sites (38). The possibility that it encounters an US citizen is 50 times lower (151). On the other hand, sporadic BL (sBL) can occur worldwide: included are those cases, with no specific geographic or climatic association. These are 1-2% of the lymphoma in adults and up to 40% of lymphoma in children in Western Europe and in the U.S.. The most common site of involvement is the abdomen - especially the ileocecal area (the junction between the small bowel and colon). Other sites like the ovaries, kidneys, omentum and the Waldeyer's ring may also be affected (48). Immunodeficiency-associated Burkitt's lymphoma refers mainly to those patients infected with the human immunodeficiency virus (HIV) but also to allograft recipients (183) and individuals with congenital immunodeficiency.

Common to all BLs are the chromosomal translocation of the *c-myc* proto-oncogene and it is considered to be the cancer-initiating event in these diseases. The translocations juxtapose the *c-myc* locus (8q24) with the immunoglobulin heavy chain gene (IGH) on chromosome 14, or with the kappa light chain gene (IGK) on chromosome 2 or the lambda light chain gene (IgL) on chromosome 22 (t(8;14)(q24;q32) or its variants t(2;8) and t(8;22)) (97). Most cases of DNA breakpoints in BL are found in rearranged VJ regions or in the S regions of the IgH loci. It has been demonstrated that in activated germinal center (GC) B cells chromosomal translocation is mediated by aberrant somatic hypermutation or class switch recombination in the presence of the activation-induced cytidine deaminase (AID) (56, 123). Thus, the formation of BL cells characteristics has its origin in GCs. As a consequence of the *c-myc*/Ig translocation, transcription of the *c-myc* locus is under the control of the active Ig locus, which results in deregulation and constitutive expression of *c-myc* RNA and, therefore, becomes an oncogene. This translocation, which is a hallmark of BL, drives cells into the cell cycle and leads to uncontrolled proliferation and cell growth, but also to apoptosis and senescence

Several pathogens were shown to contribute to BL development – such us EBV, *Plasmodium falciparum* and HIV and will be elucidated in the following paragraph.

EBV could be associated with BL to varying degrees, but its exact role in the development of BL remains elusive and is still an issue of extensive research (44). In the case

of eBL it was found that almost 100% of the cells are associated with EBV (3). The identification of EBV genomes in all tumor cells (117) indicates that the progenitor tumor cell was already infected with EBV. This supports the notion that EBV plays a role at an early stage of tumorigenesis. Furthermore, children in Uganda, with higher baseline titers to EBV antigens, are at a higher risk of developing eBL (167). The expression pattern of viral genes in eBL was found to be restricted to EBNA-1, EBER-1 and EBER-2 and BARTs (16). Since the viral antigens required for immortalization are lacking, and neither EBNA-1, nor the EBERs and the BARTs display distinct oncogenic properties that might explain EBV's contribution to the pathogenesis of BL, it is still under debate as to how EBV may directly contribute to tumor growth (16). EBNA-1, which is expressed in all EBV-positive tumors, was found to be required for episomal replication and partitioning of the viral episomes onto daughter cells. In the absence of EBNA-1, EBV can also immortalize primary B cells, but in its absence the efficiency of B cell immortalization is decreased by at least 10'000-fold (71). Expression of EBNA-1 was suggested to be critical for the continued survival of EBV-associated BL by inhibiting apoptosis *via* expression of p53 (91). Studies on transgenic mice expressing EBNA-1 showed a predisposition to develop B cell tumors (180).

Evidence that EBERs are implicated in enhanced tumorigenicity and resistance to apoptosis comes from transfection studies in the EBV-negative Akata BL cell line. Akata cell clones expressing EBER restored the malignant phenotype, resistance to apoptosis and a upregulation of bcl-2 protein expression (98). Other Akata transfection studies have also shown enhanced survival which was either mediated by virus-induced upregulation of the TCL1 oncogene (96) or through induction of IL-10 cytokine expression by EBERs (159). In addition, a binding site for MYC has been found in the promoter region of EBER1, and was associated to play a specific role of EBV in lymphomagenesis (119).

Products from BART transcripts (miRNA) were suggested to have a direct impact on the cellular gene expression pattern that supports the establishment or maintenance of latent infection (17, 149). They have been found in EBV-immortalized latency III cells and BL latency I cell lines (12, 20, 26, 150) and in BL biopsies (165, 186). Moreover, studies performed recently using BART miRNA mutants indicate that EBV miRNAs contribute to, but are not essential for, LCL formation *in vitro* (45, 46, 144)

Ironically, although a tremendous progress has been made in the understanding of the action of cellular proto-oncogenes like c-myc as well as in the biology of EBV, the molecular contribution of EBV to the pathogenesis of BL remains elusive. To investigate the oncogenic potential of EBNA-1, EBERs and BARTs, either alone or in cooperation with an activated c-

myc gene, it may be necessary to develop more robust systems that are not dependent on established tumor cell lines, but rather on primary B cells. The reconstruction of the pathogenesis of endemic BL in primary human B cells using a mouse model with humanized hematopoiesis would be the ultimate goal (16).

The malaria parasite *Plasmodium falciparum* has also shown to be implicated in the pathogenesis of eBL. The geographical distribution of the disease suggests undoubtedly an immunomodulatory role of the malaria parasite *Plasmodium falciparum* in eBL. It contributes to the increase in number of EBV-positive eBL cases and might also support the transformation of these cells. A study from Malawi reported that children expressing high levels of antibodies for EBV and carrying, at the same time, the malaria pathogen had a 13 times higher risk of developing eBL when compared to those children with low antibody levels (116). Regardless of their strong association, the relative function of the malaria parasite and EBV in the development of BL remains elusive. Additional chronic infections induced by malaria leads to polyclonal B cell activation characterized by the occurrence of hyperglobulinemia (1). The mechanism leading to this polyclonal B cell activation is still unclear.

HIV infection is another risk factor for the development of BL. The risk of HIV-positive patients to develop BL is 200- to 1000-fold higher than of HIV-negative patients (14). Immunodeficiency resulting from HIV is responsible for reactivation of EBV in latently infected B cells, which might lead to BL (116). As for malaria HIV was shown to induce polyclonal B cell activation and hyperglobulinemia (35, 103)

Hence, the main risk factor for BL development entailed by malaria and HIV seems to be polyclonal B cell activation - that may additionally be associated with higher virus titers and an increase of EBV-infected memory B cells *in vivo*.

EBV entry into B cells or epithelial cells

To enter a cell, the virus has to cross main barriers – such as the cell plasma membrane or endosomal membrane. Viruses have evolved various strategies to overcome these barriers. The keys to enter target cells are receptors. Some viruses use one specific receptor, whereas other viruses interact with at least two plasma membrane components on the cell surface. Entry of herpes viruses into the target cell is a very complex process and involves several viral ligands and host receptors interactions. Eight EBV glycoproteins are involved in EBV entry into B or epithelial cells and different receptor patterns are required depending on the cell type (74).

EBV preferentially infects B cells and its entry is mediated by binding of viral gp350/220 (BLLF1) to host cell complement receptor 2 (CR2 or CD21) (49, 52, 164) (Fig. 1). CD21 is not only a receptor for EBV entry into B cells, but is also a member of the complement system as a receptor for C3d complement fragment (138).

CD21 is the predominant receptor for EBV attachment to the B cell membrane, but a study with mutant EBV lacking gp350/220 showed that this interaction is not absolutely necessary for EBV entry into B cells (86). However, binding of gp350/220 to CD21 increased efficiency in infection of B cells (86). Monoclonal antibodies against gp350 inhibited B cell infection (172). Soluble forms of both gp350/220 and CD21 also inhibited EBV infection and bound at the same site as the CD21 antibody (164). These data underline the significance of the initial step of gp350/220 binding to CD21. On the other hand, it is most likely that other receptors are involved as well.

Common for all members of the herpesviruses family is that at least three viral glycoproteins (typically gB, gH and gL) are essential for fusion with the host cell (152). For fusion of EBV with epithelial cells these viral glycoproteins are sufficient, whereas B cell fusion requires additionally glycoprotein 42 (gp42) (Fig. 1).

Viral gp42 engaged with gHgL and gB interacts with HLA class II (107) which triggers fusion of the viral envelope with the B cell membrane (58). But from the HLA alleles, HLA-DR and HLA-DP and only some HLA-DQ alleles can bind gp42, inducing membrane fusion (95). In gp42 fusion studies it has been further demonstrated that an antibody against gH interacted with gp42 and prevented the subsequent interaction with HLA class II (107). Consequently, EBV-B cell fusion was inhibited (115). Additionally, an antibody against HLA-class II prevented interaction with gp42 and blocked infection (107).

Further studies indicated that the complex formed with soluble gHgLgp42 induced membrane fusion in the same way as gp42 alone. Interestingly, it was suggested that the cleaved and secreted form of gp42 is necessary for B cell membrane fusion (151). The soluble gHgLgp42 complex did not prevent fusion with epithelial cells. This is in contrast to gp42 only, which inhibited epithelial cell fusion. This indicates that gH, gL and gB are sufficient for efficient membrane fusion in epithelial cells. gp42 may have an inhibitory effect on the epithelial fusion process. Binding of gHgL induces a conformational change that prevents gHgL to bind to the receptor. This may be the cause of the inhibitory effect. Similarly, binding of gp42 may hinder gHgL to bind to its receptor (95).

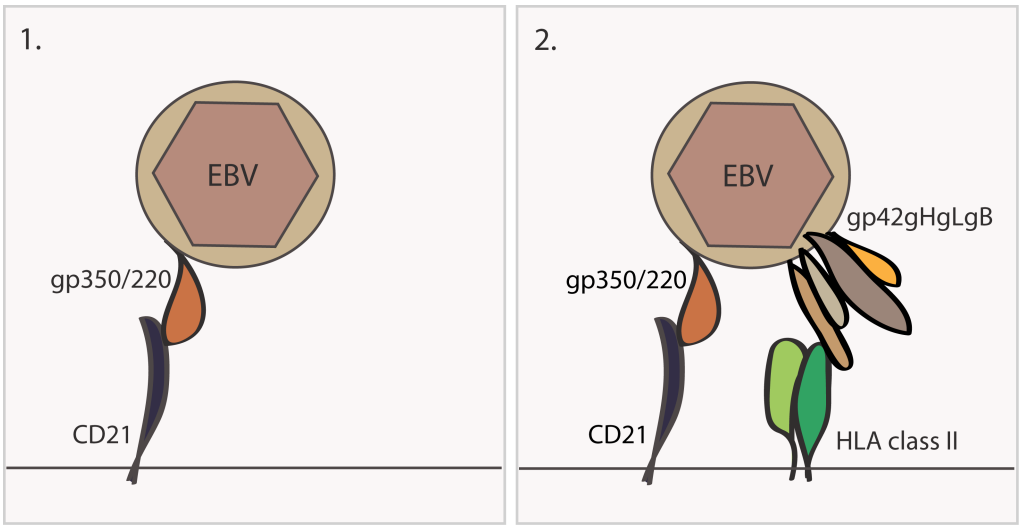

Figure 1. EBV entry into B cells.
EBV entry into B cells is initiated by binding of viral gp350/220 to cellular CD21 (1).
The following interaction of the viral complex gp42/gHgLgB with HLA-class II triggers fusion of viral envelope and B cell membrane (2).

Contribution of other glycoproteins

Interestingly, the complex gHgL has been shown to be a specific ligand for αVβ5, αVβ6 and αVβ8 integrins on epithelial cells (27). Binding of gHgL to the integrin triggered fusion of EBV with the epithelial cell membrane (28). To date, several cell membrane glycoproteins have been demonstrated to serve as co-receptors for EBV attachment to or fusion with B cells and epithelial cells (28, 43, 171). These glycoproteins have turned out to be integrins. β1 integrin was shown to interact with viral BMRF2 and is known to induce fusion of EBV and epithelial cells (171).

Our laboratory demonstrated that β1 integrin expressed on memory B cells from tonsils interacts with BMRF2. This interaction has a direct influence on downstream signaling pathways and, hence, also on the subsequent process of entry (43).

B cell development

B cells derive from hematopoietic stem cells present in the BM (66). B cell development includes several stages of differentiation in the BM before they leave for the peripheral compartments (60).

In the BM B cells pass through several distinct stages of development from the pro-B cell to pre-B cell and immature B cell (Fig. 2). During this process, rearrangement of immunoglobulin (Ig) genes results in surface expression of the pre-B cell receptor (pre-BCR)

and finally of the mature BCR. The first molecular event that leads to the mature BCRs takes place in the pro-B cell by random V(D)J gene recombination of the heavy chain. Similar rearrangement occurs with the light chain at the pre-B cell stage that includes the V and J gene. A productive rearrangement of both, the heavy and light chain, is required for positive selection of pre-B cell and progression into an immature B cell stage. To verify a functional pre-BCR capable of signal transduction, the pre-BCR is tested with a stromal cell-derived ligand, galactin-1. Pre-BCR signalling failure prevents further steps in the B cell development and the cell dies (checkpoint 1).

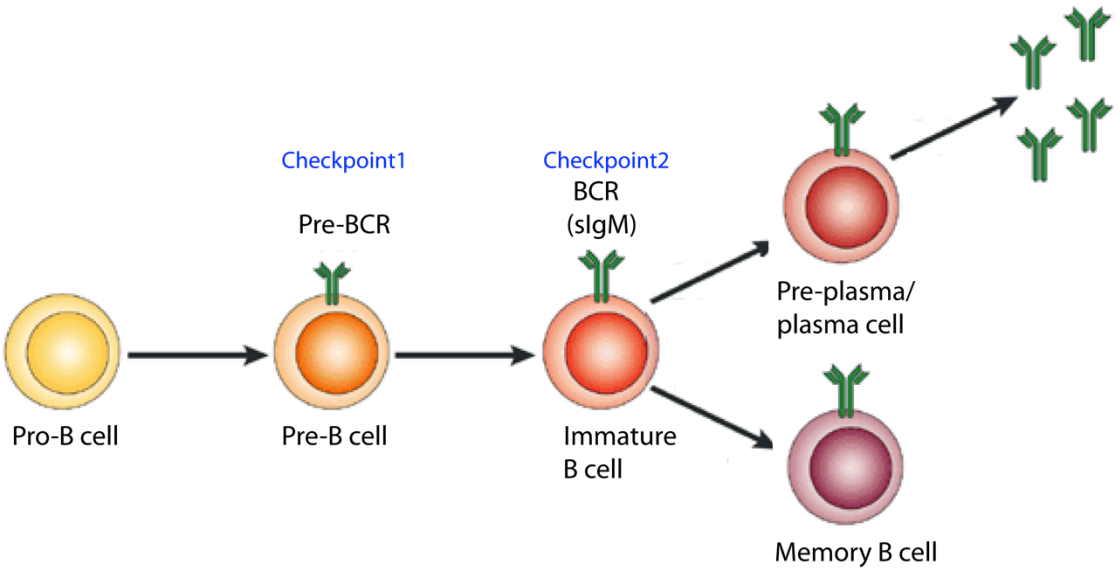

Figure 2. B cell development.
B cell development goes trough several distinct stages. In adults, B cell development starts in the BM where the rearrangement of the heavy chain of the pro-B cells and the light chain of the pre- B cells results in a functional BCR and an immature stage of the B cell. After selection (checkpoint 1 and 2) the immature B cells (naïve B cells) migrate to the spleen. Two B cell subsets of mature B cells exist: pre-plasma/plasma cells and memory B cells.
Adapted from Okkenhaug *et al*. (124).

The expression of a functional surface BCR (sIgM) leads to the progression into immature-B cells. At this stage, B cells undergo a selection process to prevent development of self-reactive cells. The autoreactivity is tested against BM-derived antigens (checkpoint 2) (143). Cells successfully completing the selection process leave the BM as fully mature naïve B cells (158). These naïve B cells eventually mature into follicular B cells or marginal-zone B cells. After encountering an antigen, B cells develop through affinity maturation and class switching into either antibody-secreting plasma cells or memory B cells.

Complement receptors CR1 (CD35) and CR2 (CD21)

Complement receptors (CR) play an important role in the interaction of the innate and adaptive immune system. The expression of CR2 (CD21) is restricted to lymphocytes and follicular dendritic cells (FDCs). CD21 has at least four ligands. On the one hand, CD21 binds to the cleavage products of C3, namely iC3b, C3dg and C3d. (81). Additionally, CD21 interacts with EBV via the surface protein gp350(49), and binds the immunomodulatory protein CD23 (7) and cytokine interferon-α (IFN-α) (6).

CD35 is expressed on multiple cell types such as red blood cells (RBC), myeloid cells and lymphocytes. (138). The murine CD21 and CD35 are encoded on the same *Cr2* locus and, thus, their mRNAs derive from the same gene by alternative splicing (100). In human and primates the *CR2* gene lost the ability to encode for CD35 during evolution. Apart from this, in humans, the exons encoding for the *CR1*-like domain still exist, but after translation the product is not functional. (85).

On primary B cells CD35 and CD21 form a complex with each other, and CD21 with CD19 and CD81. CD35 and CD19 complex does not exist (173).

Integrins

Integrins are a large family of transmembrane cell surface receptors. They consist of an α and β subunit that form heterodimers (147). These heterodimers belong to the type I transmembrane glycoproteins with a short cytoplasmic tail. To date 18 α and 8 β subunits are known in mammals and they can assemble into 24 distinct αβ receptors (108, 147, 161). They have been discovered about 26 years ago (78) and are among the best characterized adhesion receptors due to their multi-faceted functions and features.

Integrins serve as adhesion receptors and are important for contact to the extracellular environment as well as for cell-to-cell communication (161). Cell adhesion is crucial for numerous processes such as embryonic development, tissue repair, homeostasis, inflammation, cell mobilization and metastasis in multicellular organisms (79). Therefore integrin interaction with their ligands is important for attachment, cytoskeletal organization, migration, proliferation and survival. Integrins are also involved in pathological processes such as inflammation, wound healing, angiogenesis and tumor metastasis. Additionally, different microorganisms, such as viruses, can bind to integrins to get access into host cells (160).

Integrins are bi-directional signaling receptors. They can transduce the signal as classical receptors from outside-in, but also from inside-out. The outside-in signaling functions by binding to extracellular components outside the cell and transfering the signal to intracellular adapter proteins or cytoskeletal components inside the cell. The inside-out signaling across the plasma membrane mainly acts to bring the integrin into the active conformation. Both signaling pathways are accompanied by conformational changes of the integrin structure. The usual conformation of integrins on leukocytes in circulating blood is an inactive form, in which they cannot bind any ligand or transfer any signal (76). Conformational changes upon integrin activation lead to an increased ligand binding affinity and to an interaction with the actin cytoskeleton. Thus, integrins link the outside and the inside of the cells. Thereby, they transfer a signal from the extra cellular matrix (ECM) to the cytoplasmic tail and its adapter proteins to the actin cytoskeleton and activate intracellular signaling pathways or vice versa (19, 78).

Integrin structure

Integrins are non-covalently linked α / β heterodimers and they are named after their ability to integrate both the ECM and the actin cytoskeleton by signaling bidirectional. (77, 163). They are type I transmembrane glycoproteins with a large extracellular domain, a short single

membrane spanning helix and a short cytoplasmic domain (Fig. 3A) (23). They are composed of a globular extracellular head, which consists of both subunits, which are connected by two long stalks or legs to the membrane (118, 154). The α and β subunits do not share homologous sequences. But some α and β subunits show sequence similarities among their respective subunits (10).

All integrin heterodimers have several divalent cation binding sites which are important for ligand binding (169). Different divalent cations have distinct effects on ligand affinity. Mn^{2+} and Mg^{2+} support ligand binding, but latter to a lower extend. On the other hand Ca^{2+} does inhibit ligand binding (54). The divalent cation binding site and the region involved in ligand recognition are in close proximity (34, 184).

The α subunit consists of a seven-bladed β propeller, which is linked to a thigh, a calf-1, and a calf-2 domain (10, 154). The β propeller contains a domain that binds three to four Ca^{2+} cations. These Ca^{2+} binding sites have an important impact on the ligand binding activity (126, 191). An additional Ca^{2+} site is located on the Genu. Studies showed that the Ca^{2+} always occupies this site- in both bent and extended conformation. However, the precise function of this Ca^{2+} binding is still unknown (191). The half of the α subunit contains an additional αI domain (Fig. 3B). The αI domain is present in the β2 integrin subgroup of integrins, in the collagen-binding integrins belonging to the β1 subfamily (α1, α2, α10, and α11), and the αE integrin chain forming the αEβ7 heterodimer. Ligand binding induces conformational changes in the αI domain, which also alters the conformation of the β subunit ultimately affecting ligand-binding affinity (108, 155). The αI domain contains a metal ion-dependent adhesion site (MIDAS) motif. This motif harbors a Mg^{2+} ion which is coordinating ligand binding (105). Integrins that possess an αI domain can bind ligands through this domain.

The α subunit has the greatest influence on ligand binding specificity (108). In α subunits without an αI domain (Fig. 3C), the ligand-binding site is located at the interface between the α subunit β propeller and the β subunit βI domain. The specificity for ligand binding is determined by the α subunit although the βI domain is involved in ligand binding in αI-less integrins (155).

The β subunit consists of a plexin-semaphorin-integrin (PSI) domain, a hybrid domain a βI domain and four cysteine-rich epidermal growth factor (EGF) repeats (10). In contrast to the αI, the βI domain possesses three metal ion-binding sites. At the center resides one MIDAS, which binds Mg^{2+} and is flanked by two additional ion-binding sites. These binding sites are called synergistic metal ion-binding site (SyMBS) and adjacent to MIDAS (ADMIDAS) and both of them bind Ca^{2+} (191).

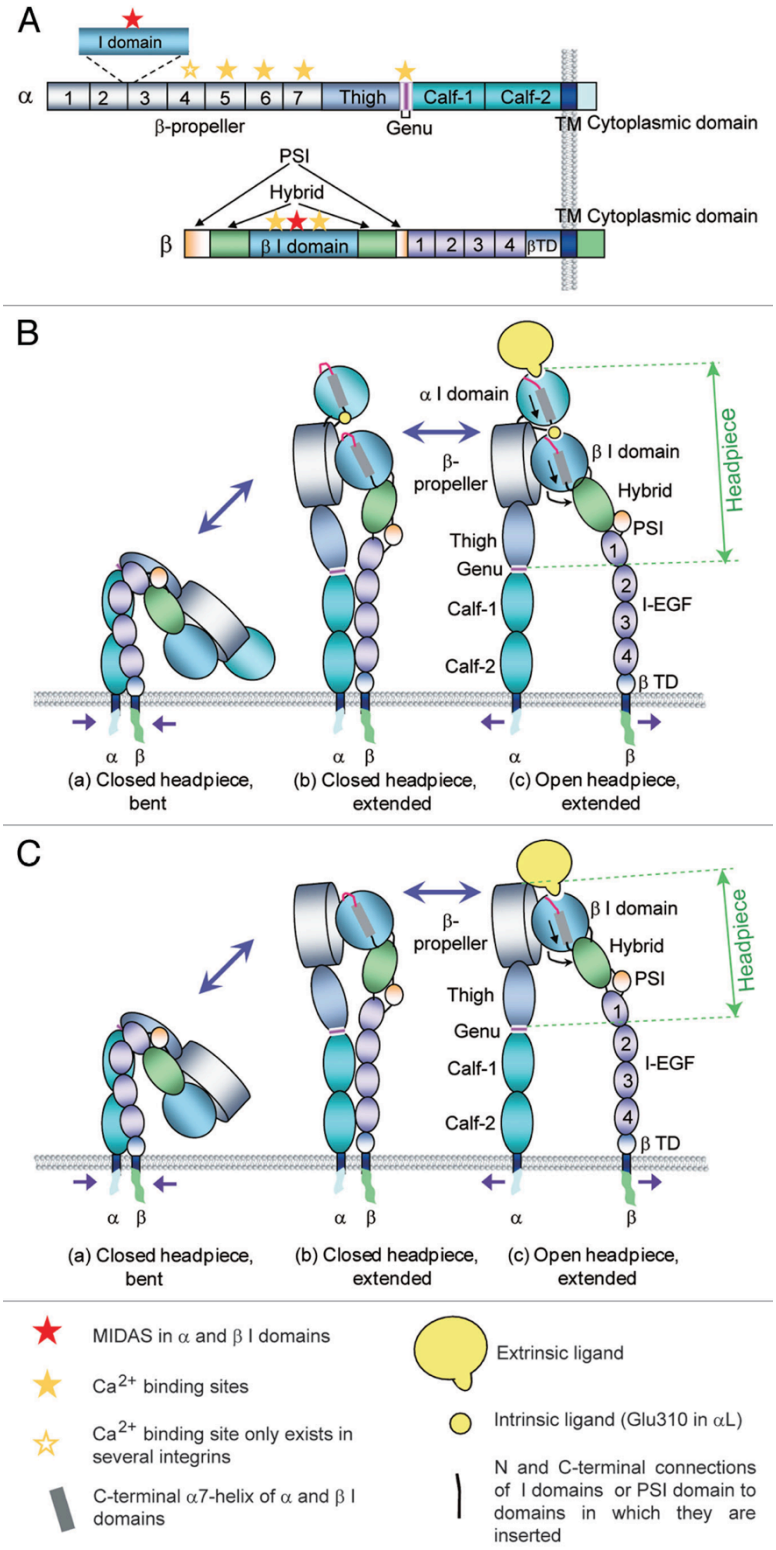

Figure 3. Schematic illustration of the integrin subunits and their potential conformations.
A) Primary structure of integrin α and β subunit. The αI domain is indicated with dashed lines. Asterisks mark the Ca^{2+} binding site in the β propeller. (B and C) Conformational changes of the αI containing domain (B) and a αI-less integrin (C) during integrin activation.
Model from Zhang *et al*. 2012 (191).

Inside-out and outside-in signaling

Depending on the extracellular or intracellular binding of ligands, the resulting conformational change and signal transmission is termed inside-out or outside-in signaling. The induced conformational changes or integrin activation include rearrangement of extracellular, transmembrane and cytoplasmic domains.

On the cell surface integrins exist usually in an inactive or non-adhesive state that is characterized by a bent or folded conformation. This is characterized by a folded conformation of the extracellular domain, a closed head and a low affinity for ligands (70, 108). To bind ligands, integrins must be in an activated state. Extracellular ligands, which are bound to B cell receptors (BCRs) or chemokine receptors, can trigger an intracellular signal to activate integrins. In this context, chemokine triggering was shown to induce a conformational change of integrins leading to an enhanced integrin affinity and was recognized as a mechanism controlling integrin-mediated adhesion (32).

Binding of an intracellular ligand, such as talin, to the cytoplasmic tail of the β subunit transforms the bent conformational state to an extended and more active form exposing the ligand-binding site. The extended form exists in two different conformations: either with a closed headpiece and with a low ligand affinity, or with an open headpiece and a high affinity for ligands. The swing out of the hybrid domain and the dissociation of the cytoplasmic domains results in an open conformation and in a higher affinity of integrins for ligands (108, 155, 162).

Inside-out signaling is characterized by binding of an intracellular ligand, such as talin or kindlin, to the integrin cytoplasmic tail. This ligand binding induces a conformational change of the extracellular domain thereby increasing the integrin affinity and clustering of the integrins in the membrane. As a consequence, the cell can regulate adhesion and migration (2). Adhesiveness can be quickly modulated by inside-out signaling (154).

Like classical receptors, integrins can also transfer signals from outside-in. Extracellular changes, such as ion concentration, can trigger a conformational change from a bent to an extended state and can activate many intracellular signaling pathways (106). Although both processes of inside-out and outside-in signaling are described separately, they are often closely linked. Integrins can be activated by intracellular signaling and, in parallel, increase ligand binding, which results in outside-in signaling. Alternatively, ligand binding can induce an intracellular signaling that causes a subsequent inside-out signaling and increases, for example, ligand binding.

Integrin ligands

Every cell type expresses a certain pattern of integrins and, depending on the combination of α and β subunits, cells can bind different ligands. Additionally, it is very characteristic for integrins to have the ability to bind multiple ligands. Moreover, many ECM ligands and cell surface proteins bind to multiple integrin receptors (73, 129). Based on their ligand-binding specificities integrins can be clustered into four subgroups. Most integrins recognize a very short motif on the ligand. The first group of integrins binds to the tripeptide sequences arginine-glycine-aspartic acid (RGD) (76). This group includes αIIβ3, all αv, α5β1 and α8β1 (Fig. 4, turquois). The amino acid sequence RGD is embedded in several molecules such as fibronectin, vitronectin, fibrinogen, the transforming growth factor-β (TGF-β) and several other ECM proteins (72). Another motif, to which integrins bind to, is the minimal key sequence LDV (leucin-aspartic acid-valine) or structurally related sequences (30, 170, 176). LDV is functionally related to RGD and both motifs are bound at the junction between the α and β dimer (72). The LDV motif is found in the alternatively spliced domain of fibronectin, the connectin sequence 1 (CS1) and in ligands such as vascular-cell adhesion molecule-1 (VCAM-1) and mucosal addressin cell adhesion molecule-1 (MadCAM-1). Integrins, such as α4β1, α4β7, α9β1 and the four members of the β2 family, interact with these ligands (Fig. 4, yellow) (72).

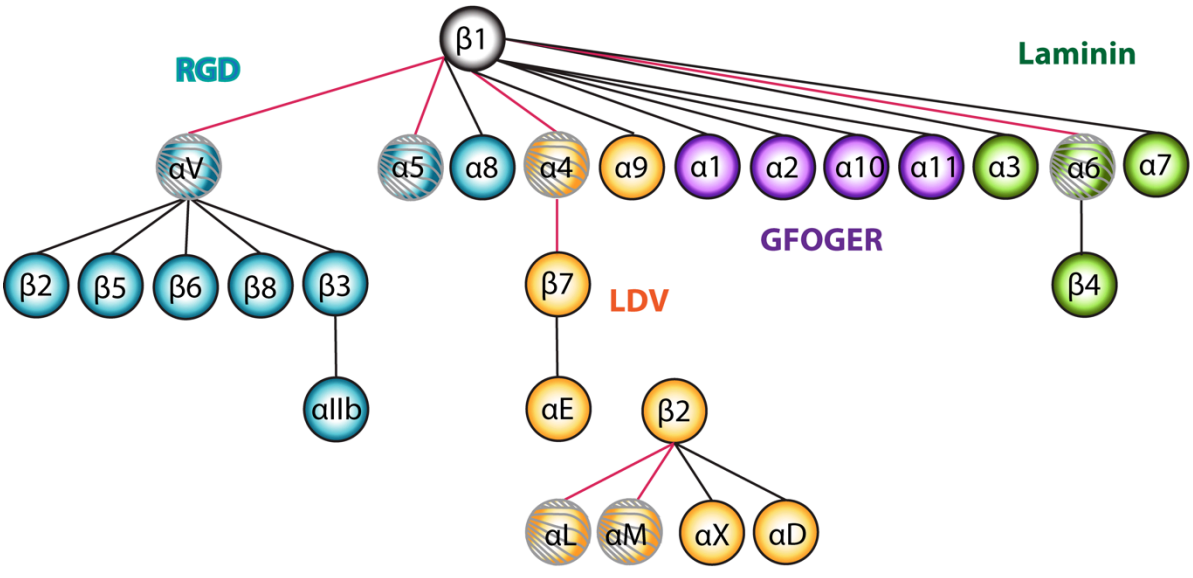

Figure 4. Representation of the integrin family.
The color code of the circles indicates the binding specificity of the heterodimer: RGD-binding integrin: turquois, LDV-binding integrin: yellow, GFOGER-binding (collagen) integrin: purple, laminin-binding integrin: green. Integrins selectively expressed in B cells have gray-striped α-subunits and are connected with red lines to the corresponding β-subunit.

A third group of integrins binds to the GFOGER motif. This is a sequence found in molecules such as collagen. This group comprises the integrins α1, α2, α10 and α11 (purple). The fourth group of integrins binds laminins. The binding integrins are formed by three β1 integrins (α3, α6 and α7) and α6β4 (green) (72).

Integrin expression on B cells

Since integrins are important for many cell processes they have also been investigated in B cells. Integrin αMβ2 was shown to be involved in B cell adhesion to the basolateral membrane of epithelial cells during transfer infection of EBV (Figure 4) gray-striped α-subunits indicate integrins expressed selectively in B cells (145). Another study demonstrated that αM integrin is associated with the BCR forming a receptor complex on the B cell surface. This receptor complex negatively regulates BCR signaling to maintain autoreactive B cell tolerance (39). The α5β1 integrin was shown to facilitate EBV attachment to memory B cells from tonsils (43). Integrin α6β1 and αVβ1 were found to be important during B cell activation on tonsillar B cells (122). In addition αLβ2, α4β1 and α4β7 are crucial for migration and development of B cells (192).

The α4 integrin subunit family

α4 integrins are expressed on several cell types including hematopoietic stem progenitor cells (HSPCs), T and B cells, natural killer (NK) cells, monocytes, eosinophils and basophils (5, 87, 133, 175). α4 integrin plays a role in leucocyte recruitment to sites of inflammation. Thus, it is also expressed on neutrophils- even though only at a low level (15). α4 integrin can dimerize with two β subunits, namely β1 and β7 (187). The heterodimers α4β1 and α4β7 bind to LDV, which is a key recognition sequence for ligand binding. Both heterodimers bind to an alternatively spliced segment of CS1 that is a domain fibronectin (FN) and to osteopontin. The vascular cell adhesion molecule (VCAM-1) is the principal ligand for α4β1. It contributes to lymphocyte homing to bronchial-associated lymphoid tissue (BALT) and is important in tissue migration during inflammation (80, 185). α4β1 can, to a minor extend, also bind to the mucosal vascular addressin cell adhesion molecule (MadCam-1). α4β7 also binds to VCAM-1, but MadCam-1 is the main ligand, which acts as a homing receptor to the intestinal Peyer's patch high endothelial venules (HEV's) (109, 187). Tethering and rolling on the endothelium can be induced by α4 and depends on rapid activation by chemokines (139). α4 integrins play a crucial role in leucocyte development and are of special importance in B and T lymphocyte development. In this regard, α4 integrins retain the hematopoietic progenitors in the bone

marrow (BM) stroma to ensure a normal early development (18). Moreover α4 inhibits apoptosis in germinal center B cells (99). In addition, crosslinking of α4 integrin prevented B cells from apoptosis and upregulated the antiapoptotic B cell lymphoma gene Bcl-XL (61). Hence, α4 integrin is essential for several biological activities and the basis of these activities can be found at the cytoplasmic tail. The cytoplasmic domain of α4 integrin is the only α subunit, which binds tightly to the signalling adapter protein paxillin. The binding of paxillin to α4 cytoplasmic domain leads to recruitment of other intracellular signalling and adapter molecules and induces the biological properties of α4 integrin (139). α4β1 integrin was also shown to engage with the tetraspan protein CD81. This engagement with CD81 modulates its function, a process termed cross talk (131).
Thus, CD81 may associate with α4β1 integrin and participate in functionally relevant interactions.

Integrins are involved in the entry of herpesvirus into the cells

Herpesviruses interact with various components on the cell surface and follow different entry routes. For instance, they have been described to enter cells by interacting with different integrins (Table 2). Binding of EBV to epithelial cells is suggested to occur via the viral envelope protein gH and/or the EBV-specific BMRF2 glycoprotein, which has an RGD integrin-binding motif (152, 153). gH is thought to bind to αVβ6 and αVβ8 integrins via a KGD motif (75) and the BMRF2-RGD domain was suggested to interact with α3, α5, αV and β1 (182). After the initial attachment of EBV to the epithelial cell surface via BMRF2 or gH/gL, a conformational change in gHgL allows, most likely, the fusion of virus and cell surface.
In B cells, so far, only α5β1 was shown to play a role in EBV infection (43). It was suggested that α5β1 was used to attach to memory B cells from tonsils, tethering EBV closer to the cell surface, and, thus, facilitating EBV infection of B cells.

Kaposi's sarcoma-associated herpesvirus (KSHV) enters target cells by utilizing a variety of receptors. The KSHV entry is mediated by interaction of viral gB with cell surface heparan sulfate and integrins α3β1, αVβ3 and αVβ5 (24, 92). α3 integrin was identified to play a crucial role in virus-induced cell fusion (174).

To enter a host cell, human cytomegalovirus (HCMV) simultaneously and independently coordinates binding to epidermal growth factor receptor (EGFR) (through gB) and the coreceptor αVβ3 (trough gH). The binding induces both EGFR-dependent PI3K and a αVβ3-dependent Scr signaling (177). β1 integrin was also suggested to be involved in

facilitating HCMV infection. But it needs to be investigated, whether β1 uses the same mechanisms as αVβ3 to enhance infection.

Herpes simplex virus 1 (HSV-1) enters cells in a receptor-dependent fashion (nectin1 or herpesviruses entry mediator (HVEM)) by the use of different mechanisms such as endocytosis, macropinocytosis/phagocytosis (55) or membrane fusion (127). A recent study considered two hypotheses: Firstly, αVβ3-integrin may bind HSV-1 and trigger endocytosis of the virus. Alternatively, αVβ3-integrin does not interact with HSV-1, but may modify, or even suppress, a common pathway of HSV-1 entry into target cell. Hence, αVβ3 integrin would become a functional sensor against HSV infection (55).

Equine herpes virus 1 (EHV-1) is a member of the *Alphaherpesvirinae* subfamily (9, 125), and enters equine epithelial cells via direct fusion at the plasma membrane (8).

Table 2. Integrins as entry receptors for herpesviruses.

Sub-family	Genus	Integrin	Cell entry	Cell type
γ-herpesvirinae	HHV-4; EBV	β1 (43), αVβ1, α3β1, αVβ1 (182), αVβ6, αVβ8 (75)	Attachment, fusion	B cells, epithelial cells
γ-herpesvirinae	HHV-8, (KSHV); Kaposi's sarcoma-associated herpesvirus	α3β1, αVβ3, αVβ5 (24, 92, 174)	Fusion	Endothelial, B, fibroblasts, epithelial cells, DCs, monocytes
β-herpesvirinae	HCMV; Human Cytomegalovirus	α2β1, α6β1, αVβ3 (47, 177)	Post-attachment	Fibroblasts
α-herpesvirinae	HSV-1: Herpes simplex virus -1	αVβ3, αVβ8 (55, 127)	Endocytosis	Epithelial cells, cells of the cenral nervous system
α-herpesvirinae	EHV-1: Equine herpesvirus-1	α4β1 (8)	Fusion	Equine epithelial cells

Gene analysis by loss- and gain-of-function studies

A lot of knowledge about mammalian cell regulation has been achieved with the aid of loss- and gain-of-gene function studies. For this purpose, exogenous genetic material, DNA and RNA can be introduced transiently or stably by transfection.

A very powerful tool has emerged from the discovery of the cellular process of RNA silencing (RNAi). RNAi is a post-translational process triggered by introduction of double-stranded (ds) RNA, which initiates the degradation of homologous RNA resulting in reduced protein expression (50). Long endogenous dsRNAs initiates RNAi by activating the RNase III family member termed Dicer (13). The Dicer binds and cleaves dsRNA into short 21 to 23 long nucleotide fragments, called small interfering (si) RNAs. The siRNA is taken up by the RNA-induced gene silencing complex (RISC) with the assistance of Argonaute 2 (Ago2). In

the RISC, the passenger strand of the siRNA duplex is degraded while the other strand, the guide strand, directs the activated RISC to the complementary sequence in the target mRNA. The Ago2 component of the RISC is a ribonuclease which cleaves the target mRNA resulting in silencing of the corresponding gene and shutting down of protein synthesis (42). Two approaches have evolved to take advantage of the RNAi machinery to shut down gene expression: (i) use of siRNA or (ii) expression of small-hairpin RNA (shRNA) that are cleaved to active siRNA. The stable transfection of shRNA can be used for effective long-term silencing. In some cases it can cause cytotoxicity due to competing cellular factors, resulting in limitation of the latter (4, 57). On the other hand, chemically synthesized siRNA may silence host gene expression also very efficiently without misbalancing host cellular factors. Although preclinical experiments have shown their effectiveness, the features of siRNAs' pose a challenge in delivery and uptake into cells. The plasma membrane is a significant barrier for siRNA uptake. The negative charge and the hydrophilicity of siRNA molecules prevent them from crossing biological membranes (42).

Unfortunately, no single transfection method can be applied for all types of cells. Facilitation of siRNA uptake is achieved with various approaches, because cellular cytotoxicity and transfection efficiencies vary dramatically - depending on the reagent, protocol and cell type being utilized.

Therefore, numerous transfection methods, such as liposome transfection, calcium phosphate precipitation, electroporation, microinjection and magnetofection are used (Table 3).

Recently, a new RNA-based method was described, CRISPR interference (CRISPRi) for targeted silencing of transcription in bacteria and humans (104). The CRISPR (clustered regularly interspaced palindromic repeats) and CRISPR associated (CAS) system is derived from the *Streptococcus pyogenes* and provides protection from invading viruses and plasmids. Bacteria harbouring CRISPR/CAS loci target foreign DNA with a short complementary single-strandet RNA (CRISPR RNA or crRNA) that localizes the CAS9 nuclease to the target DNA sequence (156).

While RNAi must target many copies of messenger RNA in order to silence gene expression, CRISPR-CAS silences gene expression already at the DNA level. This complex causes a double-stranded break at the desired DNA sequence. Several groups have so far demonstrated that the CRISPR/CAS9 system is a very efficient tool in various species (25, 29, 31, 53, 69, 88, 110, 146, 190).

Table 3. Conventional transfection methods.

Class	Methods	Advantages	Disadvantages	Examples	Refs
Biological	• Virus-mediated	-High efficiency -Easy to use -Effective on dissociated cells, slices, and *in vivo*	-Potential hazard to laboratory personnel - Insertional mutagenesis -Immunogenicity -DNA package size limited	Herpes simplex, Adeno virus, Adeno-associated virus, Vaccinia virus, Sindbis virus,	(59, 128, 137, 168, 181)
Chemical	• Cationic polymer • Calcium phosphate • Cationic lipid	-No viral vector -High efficiency -Easy to use -Effective on dissociated cells and slices -Plenty of commercially available products -No package size limit	-Chemical toxicity to some cell types -Variable transfection efficiency by cell type or condition -Hard to target specific cells	DEAE-dextran, polyethyleneimine polybrene, calcium phosphate, lipofectin, DOTAP, lipofectamine, CTAB/DOPE, DOTMA X-tremeGENE	(67, 140, 178)
Physical	• Direct injection • Biolistic particle delivery • Electroporation • Laser-irradation • Sonoporation • Magnetic nanoparticles	-Simple principle and straightforward -Physical relocation of nucleic acids into cell -No need for vector -Less dependent on cell type and condition -Single-cell transfection	-Needs special instruments -Vulnerable nucleic acids -Demands experimenter skills, laborious procedure	Micro-needle, AFM tip, GENE Gun, Amaxa Nucleofector, NEON, phototransfection, Magnetofection	(11, 41, 82, 83, 93, 111, 113, 120, 141, 142, 148, 188)

Adapted from Kim *et al*. 2010 (94).

Transfection methods have developed rapidly, even within one organism. Many new products are launched each year with improved efficiency and less cytotoxicity. Consequently, we have many options to choose from, fitting well into our experimental or clinical needs. Future studies should focus on the *in vivo* safety of the various delivery systems for clinical application, such as the RNAi-based cancer therapeutics.

Loss-of-function studies alone are usually insufficient to deduce gene function. Additional genetic approaches such as gene overexpression are needed.

Foreign genetic material, such as plasmid DNA (pDNA) can be introduced transiently or stably. Cells transiently transfected with pDNA receive the pDNA and express it before integration into genomic DNA takes place. But with every cell cycle the pDNA gets diluted and, consequently, will disappear from the cell population over time.

This is in contrast to stable transfection of pDNA where a stable cell line is generated. The stable transfection is a permanent integration of pDNA into the genome of target cells. The introduced foreign DNA is expressed by the cells continuously even after many generations of cell divisions.

Usually, an expression plasmid is integrated randomly into the genome of the target cell. The random integration sometimes leads to silencing of the transgene. This drawback initiated the development of several strategies to overcome the negative effects of random integration. Strategies such as site-specific, homologous and transposon-mediated integration are used, but require the expression of integration enzymes or additional sequences within the plasmid (84, 90).

Overexpression studies have several advantages: (1) they have dominant effects and, thus, can be performed readily in diploid organisms; (2) they provide functional links even for redundant gene; (3) they identify complementary interactions from loss-of-function screens (132).

Subject of Investigation

This dissertation investigates the involvement of integrins in EBV infection of B cells. The following topics address the primary focus of this thesis:

1) **The role of α4 integrin in EBV entry into B cell**

   CD21 is the main receptor for EBV entry into B cells. This was tested by the use of a soluble CD21 that prevented infection of B cells. Additionally, experiments with a gp350-null EBV mutant demonstrated that EBV is still capable of infecting B cells and, hence, the existence of an additional cell surface receptor was suggested. Integrins could be involved in EBV entry into B cells since α5β1 integrin has already been shown to be implicated. We investigated the expression of integrins on B cells and tested whether overexpression of α4β1 integrin in a CD21 negative B cell line increased susceptibility to EBV infection.

2) **Investigating integrins as possible EBV entry receptors by using blocking antibodies against integrins or by gene knockdown**

   A whole set of integrins is expressed on B cells. We investigated the involvement of these integrins in EBV infection of B cells by several methods.

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3) **Separation of naïve and memory B cells from tonsils by magnetic-activated cell sorting (MACS)**

   In this dissertation, we characterized integrin expression on B cells and showed a novel role of α4 integrin in EBV entry into B cells. The investigations were done in B cell lines and future studies will be necessary to study the obtained knowledge in primary B cells. Im this context, we focused on TBCs since they are located in the tonsils and, hence, are present at the portal of EBV entry.

   Separation of naïve and memory B cells is a crucial pre-requisite for many experiments investigating this process. Therefore, we established the separation of naïve and memory B cells from tonsils by magnetic-activated cell sorting (MACS).

4) Early gene expression changes by EBV infection of B cells suggesting CDKs and survivin as therapeutic targets for post-transplant lymphoproliferative diseases

A variety of EBV viral proteins are expressed in EBV-associated lymphoproliferative diseases (LPDs), and these have been shown to influence the potential risk for lymphomagenesis.

Early molecular events, leading to B cell transformation by EBV, were investigated.

Results

1) The role of α4 integrin in EBV entry into B cells

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Experimental Infectious Diseases and Cancer Research, University Childrens Hospital of Zurich, University of Zurich

Manuscript in preparation

The γ-herpesvirus Epstein Barr virus (EBV) was demonstrated to infect B cells in a CD21-dependent and -independent manner. We have previously shown that α5β1 plays a role in EBV infection of B cells however the role of other integrins in this process remains elusive. We investigated Burkitt lymphoma (BL) cell lines and primary B cells from tonsils (TBCs) for their integrin expression. We show here that the BL cell line Raji expresses α4 integrin more abundantly than TBCs and is also more susceptible to EBV infection than the latter. Moreover, overexpression of the subunit α4 integrin facilitated EBV entry into the EBV-negative cell line Akata31, but not in the EBV negative cell line BJAB. Our data indicate that α4β1 integrin plays a role in the process of B cell infection by EBV and may contribute to enhanced susceptibility of B cell subsets to EBV infection thus promoting survival of the virus.

This work was primarily done by Patricia Krukowski.

Material and Methods for Chapter 2 + 3

Cell culture and virus.

All B cells were maintained in RPMI 1640 supplemented with 10% fetal bovine serum (FBS) (Invitrogen, Basel, Switzerland), 1% L-glutamine, and 1% penicillin-streptomycin (Gibco, Basel, Switzerland) in a humidified 5% CO_2 atmosphere at 37°C.

Primary B cells were obtained from donors undergoing tonsillectomy. Peripheral blood mononuclear cells were obtained from healthy donors. The B cell line Raji originates from Burkitt Lymphoma biopsies and is EBV positive.

HEK 293 cells carrying stably expressing wildtype EBV BAC (D2089; EBV-wt B95.8)) (kindly provided by Henri Jacques Delecluse (DKFZ, Heidelberg, Germany) have been previously described (37). Cells were maintained in DMEM, 10% fetal bovine serum (FBS), 1% L-glutamine, 1% penicillin-streptomycin and 100 µg/ml Hygromycin (LabForce, Nunningen, Switzerland).

Antibodies and reagents.

Inhibitory antibody anti-CD20 mab-1048, HLA-DR mab-Tu39 (BD Bioscience, Allschwil, Switzerland), anti-CD21 mab-AT80 (AbD SeroTec, Düsseldorf Germany), anti-integrin αL/CD11a mAb-HI111, anti-integrin β1/CD29 mAb-JB1A, anti-integrin β2/CD18 mAb-P4H9, anti-integrin α4/CD49d mAb-P4C2, anti-integrin αV/CD51 mAb-LM609, anti-integrin α6/CD49f mAb clone NKI-GoH3 (Chemicon, Millipore, Zug, Switzerland), anti-IgG mab (AbD Serotec, Düsseldorf Germany).

Anti-CD19 mAb pacific blue (PB)-LT19 (AbD Serotec, Düsseldorf, Germany, anti-CD27 mAb phycoerythrin (PE)-L128 (BD Bioscience, Allschwil, Switzerland).

Isolation of primary cells.

Preparation of tonsillar and peripheral blood mononuclear cells was done as previously described (101).

Virus preparation and infection.

Free virus in supernatant was harvested from induced 293/EBV-wt cells. 293 cells at 80% confluency were transfected in 100mm Petri dish with expression plasmids (5 µg each/dish) encoding the BZLF1 and the BALF4 gene products, using the liposome-based reagent Metafectene (Biontex, Martinsried/Planegg, Germany). After 5 hours, the transfection mixture was removed and cells were kept in DMEM supplemented with 10% fetal bovine serum

(FBS), 1% L-glutamine, and 1% penicillin-streptomycin. Three days post virus induction, virus supernatant was filtered trough a 45 μm pore size filter and stored at 4°C until use. Determination of infectious EBV particles was done as described in (40).
B cells were infected with viral supernatant of multiplicity of infection (MOI)=6. After 24 hours medium was replaced and cells were analysed by flow cytometry for EBV infection.

Real-time polymerase chain reaction (RT-PCR).

Total RNA isolation, DNAse treatment, reverse transcription and RT-PCR were performed as described earlier (102, 134).
qPCR was performed for the integrins α3, α4, α5, α6, αE, αL, αM, αV, β1, β2, β4 and β7 and integrins were normalized to the housekeeping gene hydroxymethylbilane-synthase (HMBS) and further normalized to control (depicted as $2^{\Delta\Delta ct}$). Total RNA was isolated after 24 hours post splitting the cells. All subunits and HMBS were analyzed using primer/probe on demand (Assay-on-demand gene expression product; Life Technologies, Zug, Switzerland).

Flow cytometry.

Flow cytometry was performed using a FACS Canto II flow cytometer (Beckman Coulter, Nyon Switzerland). Flow cytometry data were analyzed with FlowJo.

Knockdown of α4 integrin or CD21 expression with siRNA.

Raji B cells were split 24 hours before transfection. On the day of transfection, Raji cells were either transfected with negative control or with α4 integrin specific or control siRNA (Qiagen Allstars Negative Control siRNA Cat # 1027284) using liposomal method with HiPerFect (Qiagen, Basel, Switzerland), XtremeGENE (Roche, Rotkreuz, Switzerland) or NEON transfection systems (Life Technologies, Zug, Switzerland).
HiPerFect: 24 hours prior to transfection, cells were split. 200 000 Raji cells were transfected with 750 ng of siRNA in 100 μl culture medium without serum and 6 μl HiPerFect Transfection reagent. After 6 hours culture medium with antibiotics was added. 48 hours post transfection cells were analyzed by flow cytometry.
XtremeGENE: 200'000 cells were transfected with different ratios of transfection reagent and siRNA (10:2, 2.5:0.5 and 1:0.2). 48 hours after transfection, cells were harvested for detection of surface integrin expression by flow cytometry.

shRNA introduction with lentiviral particles.

$1x10^5$ cells/mL in 1ml were transferred into sterile 15 ml Falcon tubes.

MISSION lentiviral particle solution was added to the cells such that the final MOI was 4.0 per Falcon tube.

Falcon tubes with cell-virus mix were centrifuged at 800 x *g* for 60 minutes at 32 °C. Subsequently the virus-containing medium was aspirated and disposed. Each cell pellet was resuspended in 1 ml of completed RPMI by gently pipetting the pellet up and down, and each resuspended pellet was transferred to a well in a 12-well culture plate. The plate was incubated for two days in the incubator at 37°C and 5% CO_2. After 2 days, media was removed and replaced with 2 ml RPMI complete media containing puromycin (Raji: 800 ng/mL; BJAB: 900 ng/ml.

Cells were maintained in puromycin until the non-transduced negative control cells have died as determined by inspection or Trypan Blue staining.

Lentiviral transduction particles are specified in the appendix.

The following two chapters (2 + 3) include additional unpublished results.

2) Inhibition of EBV entry into B cells by targeting integrins with blocking antibodies or gene knockdown

Blocking of CD21 and HLA-DR, but not integrins by specific antibodies, reduced EBV infection of B cells

To determine whether integrins play a role in EBV entry into B cells (74, 153), we performed experiments using blocking antibodies hindering EBV binding to integrins on Raji cells and TBCs.

In order to titrate the antibodies used for the blocking experiments, Raji cells were incubated with different concentrations of specific antibodies for CD21, and control antibodies specific for CD20 and IgG. In a next step, the cells were spinoculated with GFP-tagged EBV-wt and analyzed by flow cytometry for the percentage of positive cells and mean fluorescence intensity (MFI) (Fig. 5A and B).

Incubation with an antibody specific for CD21 and with a concentration of 10 μg/ml displayed the highest reduction of susceptibility to EBV infection, demonstrating a significant reduction of GFP expression (30%). These results are comparable to previous publications (43). Control antibodies specific for CD20 or IgG had no effect at the different concentrations used. A higher antibody concentration (25 μg/ml) showed a toxic effect on Raji, likely caused by sodium azid.

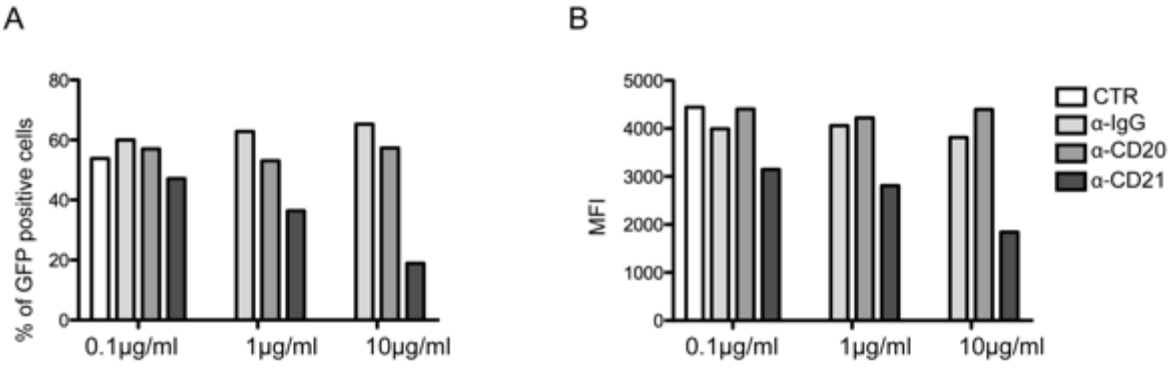

Figure 5. Titration of blocking antibody against CD21 on Raji cells.

Raji cells were incubated with anti-CD21 or control antibodies anti-IgG1 or anti-CD20 (0.1 μg/ml, 1 μg/ml and 10 μg/ml) before spinoculation with EBV. Infected cells were identified by flow cytometry. (A) Depicted are percentages of GFP positive Raji cells and (B) mean fluorescence intensity (MFI).

To test whether integrins are required for EBV entry into B cells, we used blocking antibodies directed against α4, αL, αV, β1 and β2 integrin and tested EBV binding to TBCs and Raji cells. The blocking antibodies did not exhibit any inhibitory effect on EBV infection neither in tonsillar memory ($CD191^{+}$ $CD27^{+}$), naïve ($CD19^{+}CD27^{-}$) B cells (Fig. 6A) nor in Raji cells (Fig. 6B). Incubation with control antibody specific for CD21 resulted in a reduction of GFP positive TBCs of about 65% and 60% Raji cells. As expected, incubation with control antibody directed against CD20 had no effect.

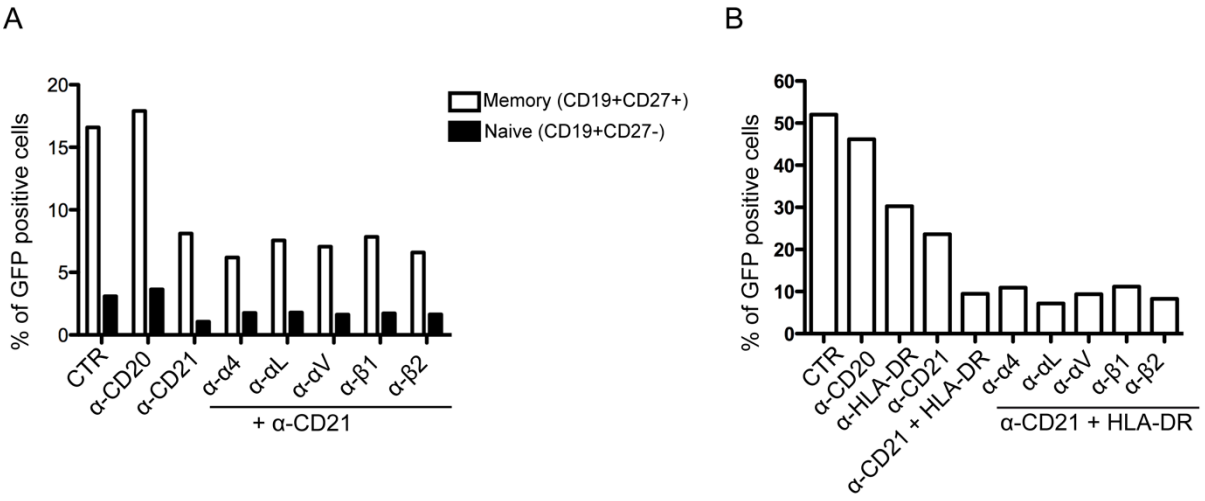

Figure 6. α-CD21 and α-HLA-DR blocking antibodies inhibited EBV infection but not integrin function blocking antibodies.

(A) TMCs were incubated with blocking antibodies against CD21, α4, αL, αV, β1 and β2 integrins or control antibodies before spinoculation with EBV. After 72 hours GFP expression was measured by flow cytometry. (B) Raji cells were incubated with blocking antibodies against CD21, HLA-DR, integrins or control antibodies before infection with EBV by spinoculation. Infected cells were identified by flow cytometry for GFP. One representative experiment is shown. CTR = control cells, only infection.

In Raji cells, antibodies specific for CD21 and HLA-DR showed the highest reduction in susceptibility to EBV infection. The antibody specific for CD21 displayed a reduction in susceptibility to EBV infection of 60% and HLA-DR 40% (Fig. 6B). The combination of these two antibodies resulted in a reduction of susceptibility to EBV infection of 70%. On the other hand, antibody specific for α4, αL, αV, β1 and β2 in combination with antibodies specific for CD21 and HLA-DR had a minimal effect on susceptibility to EBV infection.

Gene-knockdown by transfection of B cells

To investigate the function of a certain gene, gene-knockdown by RNA interference is a very powerful tool. Several methods to deliver siRNA are available, but transfection of B cells is very difficult in terms of efficiency and by far not all methods are suitable.

Because α4 integrin was shown to be involved in EBV entry into B cells and is expressed in all cells at a high level (described in chapter 1) we silenced α4 integrin in Raji cells by transfection with siRNA against α4 integrin. We tested chemical, physical and viral methods to deliver siRNA into B cells.

Chemical methods:

Chemical transfection is the most commonly used method and was the first method we tested. We tested two lipid based transfection reagents, which differed in the charge of the lipids forming complexes with the siRNA. The first reagent used (HiPerFect) contained cationic and neutral lipids, whereas the second contained only neutral lipids (XtremeGENE).

We transfected Raji cells with HiPerFect (Qiagen), in which the lipid-siRNA complexes were delivered in positively charged liposomal vesicles into the cells.

Transfection of a fluorochrome tagged control siRNA (Alexa Fluor 488) resulted in a transfection efficiency of 50% (Fig. 7A). After 24 hours, 48 hours or 72 hours no significant and specific down of neither GAPDH (Fig. 7B) nor α4 integrin mRNA (Fig. 7C) was achieved in comparison to non-target siRNA.

The second lipid transfection reagent XtremeGENE (Roche) contained only neutral lipids and demonstrated only a low transfection efficiency of 7% (Fig. 7D). Moreover, no down-regulation of neither GAPDH nor α4 integrin mRNA in comparison to non-target siRNA was observed (data not shown).

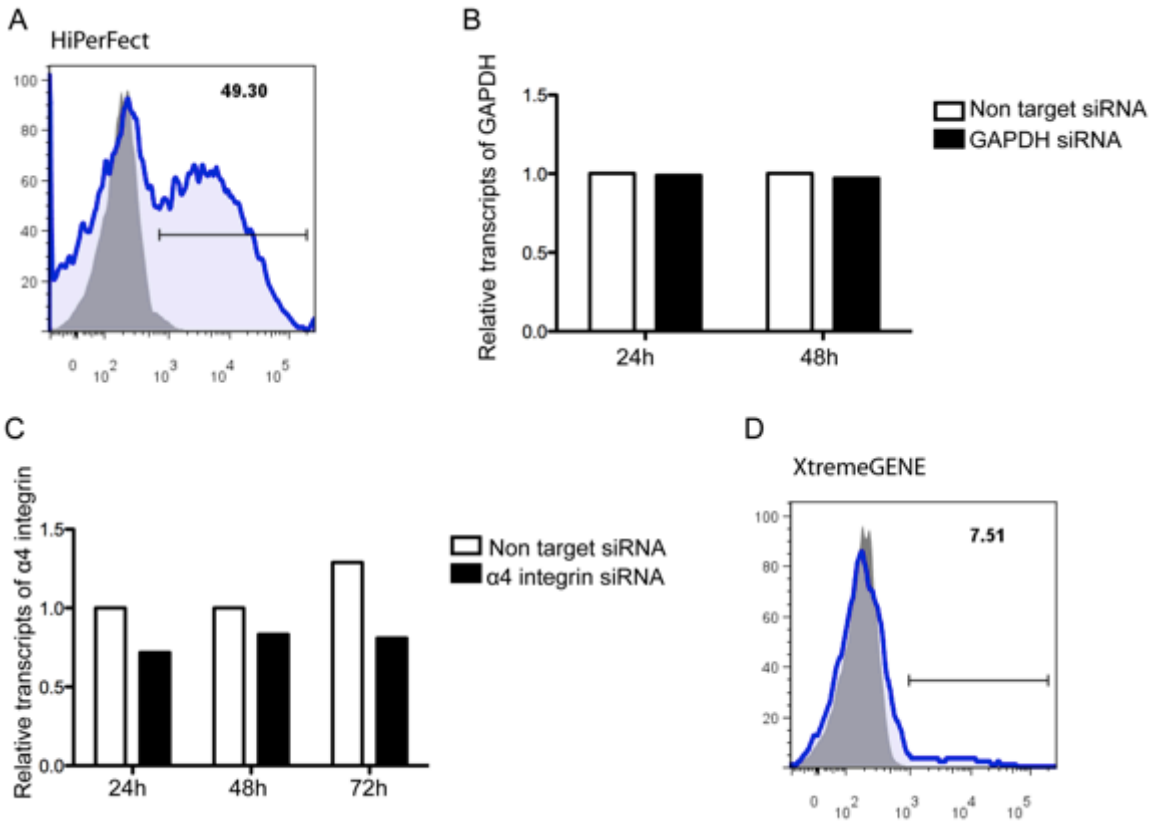

Figure 7. Liposome-based transfection of Raji cells.
Raji cells were transfected with two liposome-based transfection reagents. (A) HiPerFect (Qiagen): Raji cells were transfected with Alexa Fluor 488 tagged siRNA. Flow cytometry data illustrate the histogram analysis of Alexa Fluor 488. Numbers on the plot indicate the percentage of positive cells for Alexa Fluor 488. (B) Raji cells were transfected either with non-specific siRNA or with siRNA specific for GAPDH. Results are expressed as $2^{-\Delta\Delta ct}$. (C) Raji cells were transfected with either non-specific siRNA or specific siRNA against α4 integrin. Results are expressed as $2^{-\Delta\Delta ct}$. (D) XtremeGENE (Roche): Raji cells were transfected with a second liposomal transfection reagent. Numbers on plot indicate the percentage of positive cells for the Alexa Fluor 488 tagged siRNA.

Physical transfection methods:

Physical transfection methods, like electroporation of magnetofection, are based on delivery of exogenous material through the membrane by physical forces.

We tested two different approaches to evaluate the best physical transfection method, namely electroporation and magnetofection.

1) Electroporation: Electroporation is a widespread method used for molecule delivery into target cells. In general, electroporation has been known to be less efficient than other methods, especially when compared to viral transfection methods. But, on the other hand, it has advantages in terms of the larger capacity of DNA insertion (89).

We first determined the optimal electroporation conditions which was defined as the highest detection of the Alexa Fluor 488 labeled control siRNA and the highest cell viability rate. Electroporation (Neon transfection systems by Life Technologies) of Raji cells resulted in high transfection efficiency with the Alexa Fluor 488 tagged control siRNA. As detected by flow cytometry, 93% of the cells were positive for the Alexa Fluor 488 siRNA in comparison to non-transfected cells (Fig. 8A). To test the efficiency of silencing at the mRNA level we transfected a validated siRNA against GAPDH. As measured by RT-PCR, transfection with this siRNA resulted in >85% gene silencing compared to non-specific siRNA (Fig. 8B). Thus, B cell transfection by electroporation is highly efficient.

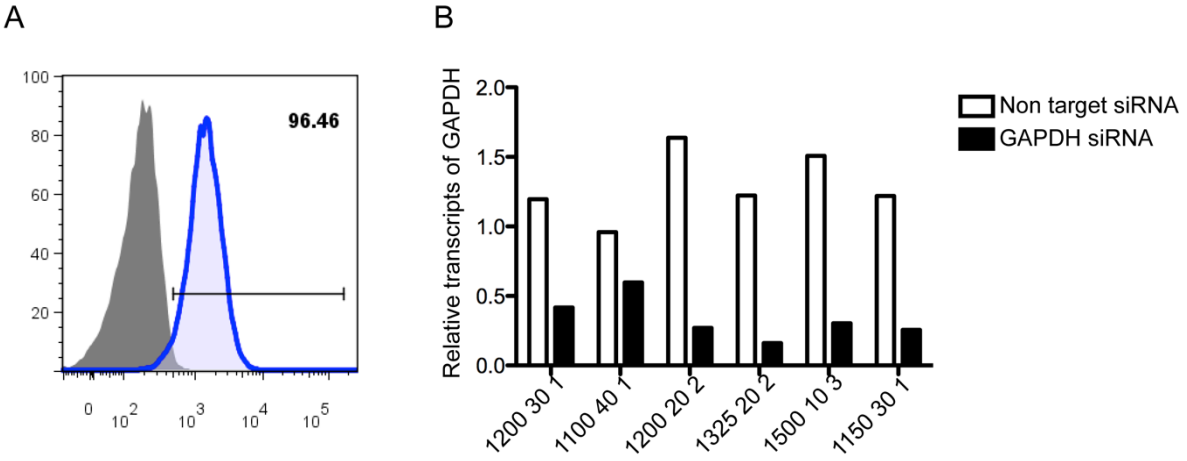

Figure 8. Optimization of electroporation conditions with Raji cells.
(A) Raji cells were electroporated (Neon) with the parameters 1325-20-2 (pulse voltage-pulse width-pulse number). Cells were analyzed by flow cytometry 48 hours after electroporation and data are depicted as histogram. Numbers on plot indicate the percentage (%) of positive cells for Alexa Fluor 488. The parameters 1325-20-2 resulted in the highest percentage of positive cells for Alexa Fluor 488. (B) Raji cells were transfected with either non-specific siRNA or with siRNA specific for GAPDH and the relative transcripts were measured by RT-PCR. Various parameters for electroporation were tested and are represented in numbers below the bars. Results are expressed as $2^{-\Delta\Delta ct}$.

2) Magnetofection:

This technology was first used in 2002 (141) and is based on the use of magnetic nanoparticles.

In order to compare the two physical methods, namely electroporation and magnetofection, Raji cells were transfected with siRNA against α4 integrin with both methods in parallel. The viability of magnetofected Raji cells was very low (0-5%) (data not shown). This is in

contrast to the manufacturer's information, which states that one of the advantages of magnetofection is high cell viability. Efficiency of silencing was tested with siRNA specific for α4 integrin in comparison to the mRNA of GAPDH. Electroporation with siRNA specific for α4 integrin reduced mRNA expression of α4 integrin by 50%, whereas magnetofection with the very same siRNA reduced the α4 integrin expression rate only about 10% (Fig. 9). Although the magnetofection experiment was performed only once, the results obtained showed that the protocol must be improved. The cell line BJAB was tested as well, but none of the cells survived (data not shown).

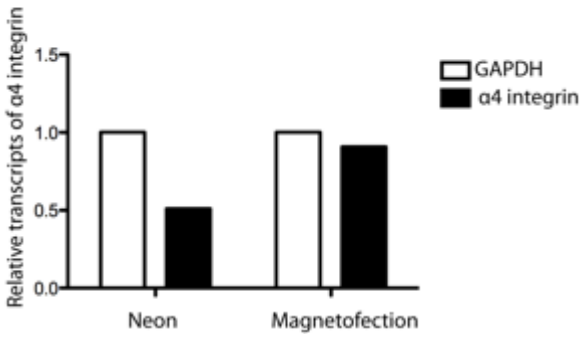

Figure 9. Transfection of Raji cells in parallel with electroporation (Neon) and magnetofection. Bar graph showing relative α4 integrin transcripts in Raji cells. Raji cells were transfected with siRNA specific for α4 integrin or non-target siRNA. The resulting relative transcripts of α4 integrin were normalized to GAPDH. Results displayed represent one experiment.

Biological methods: Virus-mediated transfection

Virus-mediated transfection is considered to be highly efficient and is also termed viral transduction. We silenced α4 integrin and CD21 in Raji and BJAB cells by viral transduction. We generated stable cell lines with Raji and BJAB cells with lentiviruses encoding non-target control shRNA, and shRNA targeting B2M (beta 2 microtubulin), α4 integrin or CD21. After infection, cells were selected and amplified for five weeks. Non-target or B2M transfected cells did not survive, most probably because of insufficient infection by the lentiviruses. Most of the Raji cells died rapidly after puromycin selection, indicating an inefficient transfection by lentiviruses as well. Therefore, only a few clones could be analyzed by flow cytometry (see Fig. 10A and B). Nevertheless, α4 and CD21 cells were analyzed for surface marker expression by flow cytometry.

Integrin α4 protein expression in BJAB and Raji was not reduced in virally transduced cells in comparison to parental cells (Fig. 10A). Also the expression of CD21 was not reduced (Fig. 10B).

In summary, an efficient silencing with specific siRNA against GAPDH could be achieved, but not with siRNA against α4 integrin. In addition, silencing with shRNA was not efficient, but could be possibly improved with changes in the infection procedure.

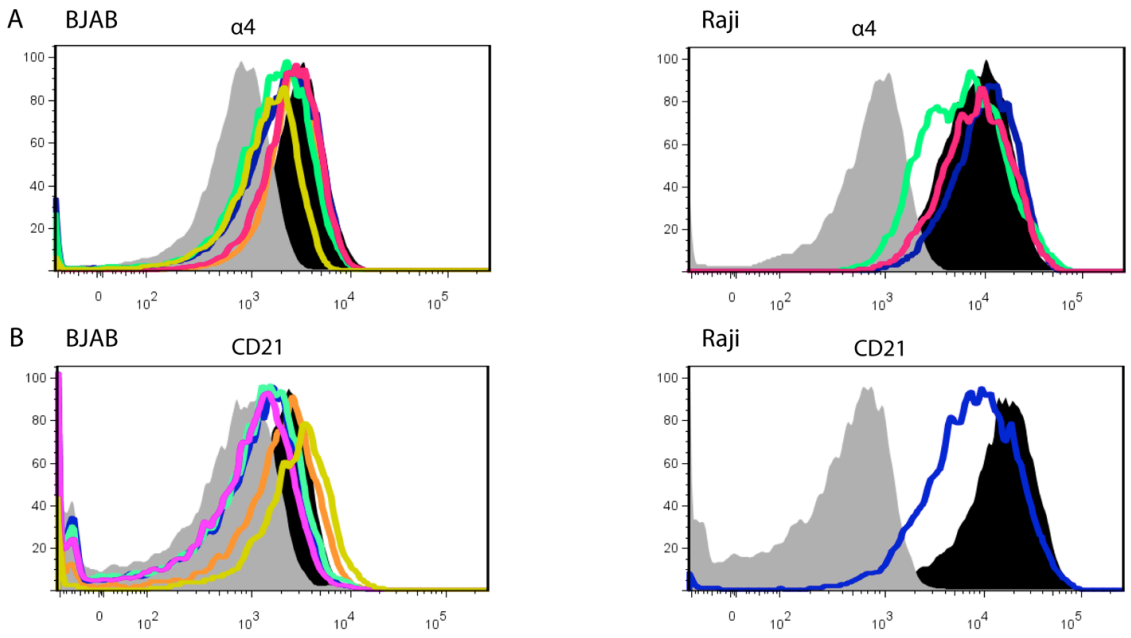

Figure 10. BJAB and Raji cell transduction with lentiviral particles encoding shRNA against α4 integrin or CD21.

BJAB or Raji cells were transduced with lentiviral particles (MOI=4) harboring either pLK0.1 encoding shRNA against α4 integrin (A) or CD21 (B). α4 integrin or CD21 expression was measured by flow cytometry five weeks after viral-mediated transfection. Shown is a representative histogram of the cells stained with α4 integrin or CD21 antibody. Wildtype cells stained with specific antibody are in black and isotype control in grey. Histograms in heavy lines represent different shRNA sequences (S): S1-orange, S2-blue, S3-green, S4-pink, S5-yellow.

3) Separation of naïve and memory B cells from tonsils by MACS (Magnetic-activated cell sorting)

In this dissertation (topics 1+2), we characterized integrin expression on several B cell lines and showed a novel role of α4 integrin in EBV entry into B cells. In future studies it will be interesting to investigate whether the information obtained from B cell lines can be confirmed in primary B cells. TBCs are located in the tonsils where the portal of EBV entry is located portal of EBV entry is. Therefore, we decided to focus on TBCs. For future investigations of the involvement of integrin in EBV infection of TBCs a diversity of different experiments will be necessary. Experiments will include RT-PCR or gain-of-function experiments in primary B cells and, furthermore, physically separated naïve ($CD19^+CD27^-$) and memory ($CD19^+CD27^+$) B cells will be needed. To separate naïve and memory B cells, we decided to use a separation method based on magnetic-activated cell sorting (MACS) MicroBeads. These MicroBeads have been developed to separate human B cells and their subpopulations from the peripheral blood. The separation of naïve and memory B cells is based on a two-step procedure. First, B cells ($CD19^+$) are separated from the leukocytes population by a negative separation, whereof memory B cells ($CD19^+CD27^+$) are separated with a second step by positive separation.

Non-B cells were labeled with a biotin antibody cocktail including CD2, CD14, CD16, CD36, CD43 and CD235a and, thereafter, magnetically labeled with anti-biotin MicroBeads and the cell suspension was loaded onto a MACS column. The non-labeled cells (CD19-positive B cells) were collected in the flow through (Fig. 11B-D).

Subsequently, the CD19-postive cells were magnetically labeled with CD27 MicroBeads and loaded onto a MACS column. The unlabeled cells run through the column and the CD27-positive cell fraction was retained in the column. The CD27-positive cells were rinsed from the column after removing it from the magnetic field (Fig. 11B-D). After every separation step, cells were stained with antibodies against CD19 and CD27 and the purity of the cell population was determined by flow cytometry. Gates were set as depicted in Figure 11A.

In peripheral blood mononuclear cells (PBMCs), the separated memory (CD27-positive) B cells were detected by flow cytometry (Fig. 11B). In contrast to PBMCs, no CD27-positive cells could be detected in the tonsilar mononuclear cells (TMCs) after separation from the CD19-positive cells (Fig. 11C). Separation of CD27-positive cells from TMCs was successful after CD19-negative and IgD negative separation (Fig. 11D), but was not verifiable after an additional labeling and separation procedure with CD27 MicroBeads. This indicates that CD27 MicroBeads might hinder the binding of CD27 antibody to tonsillar memory B cells,

but not from the peripheral blood. Thus, separation of naïve and memory B cells from tonsils is possible by using the IgD marker.

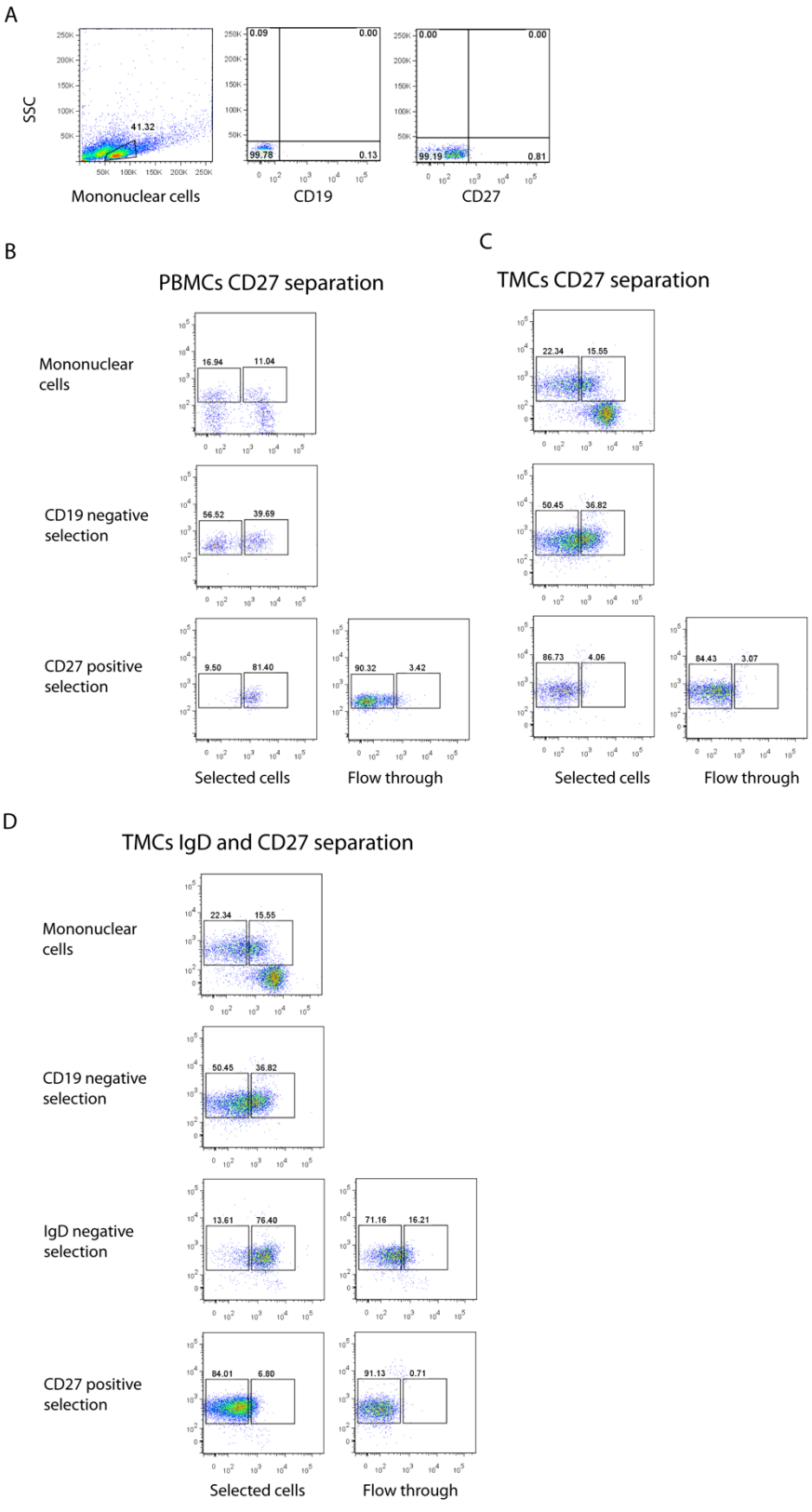

Figure 11. Separation of memory B cells from tonsils using MACS MicroBeads.
The memory B cell isolation kit was used for the isolation of memory B cells from PBMCs and TMCs. (A) Gates of CD19 and CD27-positive cells were set according to FMO (fluorescence minus one) conditions. (B) CD27 memory B cells were isolated from human PBMCS first by depletion of non-B cells and subsequent positive selection with CD27 MicroBeads. Cells were stained with antibodies against CD19 and CD27. (C) Isolation of CD27 memory B cells from TMCs with CD27 MicroBeads and cells were stained with antibodies anti-CD19 and CD27. (D) Depletion of non-B cells and IgD positive cells was done with TMC and a subsequent separation with CD27 MicroBeads. Cells were stained with antibodies against CD19 and CD27.

4) Early gene expression changes by Epstein-Barr virus infection of B cells indicate CDKs and survivin as therapeutic targets for post-transplant lymphoproliferative diseases

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Manuscript is published in International Journal of Cancer 2013

Abstract:

Lymphoproliferative diseases (LPDs) associated with Epstein-Barr virus (EBV) infection cause significant morbidity and mortality in bone marrow and solid organ transplant recipients. To gain insight into LPD pathogenesis and to identify potential effective therapeutic approaches, we investigated early molecular events leading to B cell transformation by gene expression profiling of EBV-infected B cells from tonsils by Affymetrix microarray 72 hr postinfection when the B cells hyperproliferation phase starts. Cell cycle and apoptosis were the most significantly affected pathways and enriched gene sets. In particular, we found significantly increased expression of cyclin-dependent kinase (CDK)1 and CCNB1 (cyclin B1) and of one of their downstream targets BIRC5 (survivin). Importantly, the strong upregulation of the antiapoptotic protein survivin was confirmed in lymphoblastoid cell lines (LCLs) and 71% of EBV-positive post-transplant EBV-LPD lesions scored positive for survivin. The validity of early transforming events for the identification of therapeutic targets for EBV-LPD was confirmed by the marked antiproliferative effect of the CDK inhibitor flavopiridol on LCLs and by the strong induction of apoptosis by survivin inhibition with YM155 or terameprocol. Our results suggest that targeting of CDKs and/or survivin in post-transplant EBV-LPD by specific inhibitors might be an important approach to control and eliminate EBV-transformed B cells that should be further considered.

Discussion and Future Perspectives

The α4β1 integrin was first identified as an activation-dependent receptor on hematopoietic cells. Research has transformed our understanding of this integrin and demonstrated that α4β1 integrin is a very dynamic cell surface receptor that mediates several biological activities, in particular B and T cell development and prevents GC B cells from undergoing apoptosis.

The work presented in this dissertation shows a novel role of α4β1 integrin in EBV entry into B cells.

An interesting observation is that Raji cells were more susceptible to EBV infection than primary B cells. Given that Raji cells and primary B cells show a similar expression level of the known EBV receptors CD21 and HLA-DR, we hypothesized that the increased susceptibility of Raji cells to EBV infection was due to their higher expression of α4 integrin. In particular, recent studies on EBV entry revealed that, depending on the cell type, different integrins are involved in viral entry into host cells. So far α3, α5, αV (182) and β1 integrin (171, 182) were shown to play a role in EBV infection of polarized epithelial cells, but for B cells only α5β1 has been associated with increased infection (43). Enhanced susceptibility of Raji cells to EBV infection could also be due to a more abundant expression of CD35, a recently described EBV co-receptor in B cells, (121). We confirmed that Raji cells lack CD35 (121) whereas TBCs did express CD35. Apparently, the absence of CD35 did not seem to reduce the susceptibility of Raji cells to EBV infection in comparison to primary B cells that express CD35. The higher expression of α4 integrin in Raji cells compared to primary B cells suggested that this integrin might indeed favor the infection process of B cells.

Moreover, overexpression of α4 Integrin in BL cell line Akata31, which is poorly infectable with EBV, revealed a solid increase by two-fold in susceptibility to EBV infection. Based on these results, the question arises, for which process EBV might employ α4β1: as an attachment or post attachment receptor? We propose that the cellular α4β1 can be used as an attachment receptor bringing EBV closer to the cell membrane and, simultaneously, might induce a possible signaling event in the B cell (Fig. 12-1). Binding of viral pg350/220 to CD21 is absent in the model cell line Akata31, but it does take place in B cells expressing CD21 (Fig. 12-2). The arising interaction of gp42gHgLgB with HLA-class II induces fusion of the viral envelope with the cellular membrane (Fig. 12-3). The question of whether integrins are used only as attachment molecule or whether it is necessary as a post-attachment

event (internalization, signaling etc.), has been investigated for several viruses. For example, HSV-1 was shown to use αVβ3 to fuse with the host cells (55). HCMV interacts with α2β1, α6β1, and αVβ3 integrin to induce internalization (47, 177). In the case of EBV it is depended on the cell type: for EBV entry into B cells, integrins play a role as co-receptors for attachment to B cells. But as for epithelial cells, EBV binds integrins for attachment (74) and internalization (27).

The hypothesis that interaction of EBV and α4 could induce a signaling event in B cells is an intriguing idea and further investigation might shed light on the precise function of the cooperation of α4 integrin with an EBV receptor. One study reported that binding of α4 integrin to its natural ligand VCAM-1 activates the downstream signaling and prevents apoptosis of GC B cells during their selection process (99). Thus, binding of EBV to naïve B cells would induce the α4 Integrin signaling pathway and, for the benefit of EBV, support the rescue of EBV-infected B cells.

To investigate whether EBV itself activates integrin signaling thereby enhancing infection efficiency, the phosphorylation state of FAK, PI3K upon binding of EBV to B cells should be examined.

It has been already been shown, that down stream signaling of α5β1 integrin enhances infection efficiency in tonsillar memory B cells (43). The initial step in integrin signaling involves activation of the fokal adhesion kinase (FAK) followed by downstream signaling trough phosphoinositide-3-kinase (PI3K) and the tyrosine-protein kinase Scr (c-Scr). In contrast to other alpha subunits, α4 integrin has the ability to induce signaling. The biological activities of α4 integrin depend on the cytoplasmatic domain. Proteins interacting with the cytoplasmic domain increase cell migration. Paxillin, a signaling adapter protein, binds directly to the α4 integrin subunit, but not to most othe cytoplasmic domains. Further downsteram, Paxillin directly interacts with several intracellular signaling and adapter proteins, such as the focal adhesion kinase (FAK).

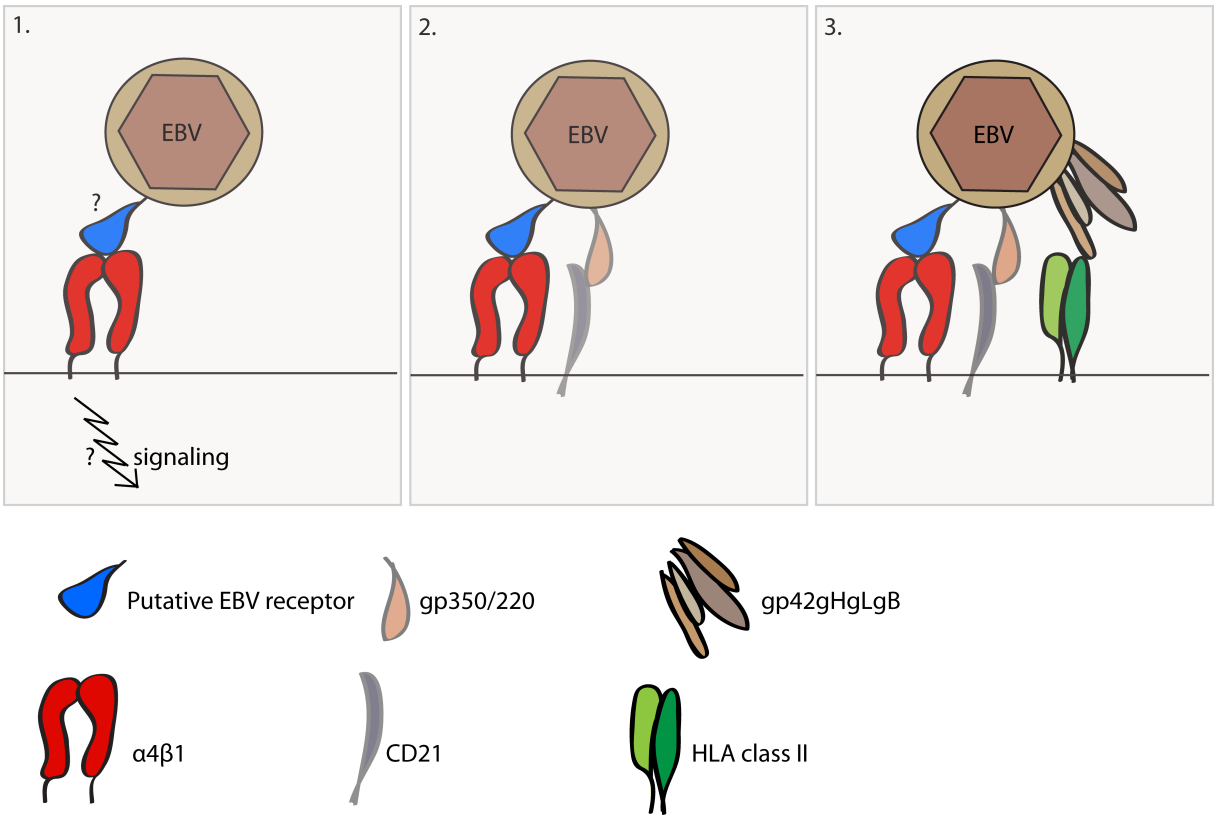

Figure 12. Putative model of EBV entry into B cells.
EBV attachment is facilitated via an unknown EBV receptor to the B cell receptor α4β1 integrin (1). This interaction brings EBV closer to the cell membrane and induces a possible signaling event (2). Subsequently, viral gp350/220 might bind to the cell surface molecule CD21. The interaction of the viral gp42/gHgL complex with HLA-class II triggers fusion of viral envelope and B cell membrane (3). The depicted interaction of the receptors CD21 and gp350/220 in (2) and (3) are grayed out and do not exist in our model cell line Akata31. In primary B cells this interaction certainly happens.

While gain-of-function experiments revealed an involvement of α4 in EBV entry into Akata31 cells, silencing experiments were more difficult to perform in B cells.

First, blocking experiments with inhibitory antibodies against integrins expressed on B cells did not result in a decreased ability of EBV to infect Raji or tonsillar B cells. Nonetheless, the control antibody against CD21 decreased EBV susceptibility to EBV infection. Interestingly, not every commercial available inhibitory α-CD21 antibody could block EBV binding to B cells. The successful inhibition of EBV entry into B cells preceded a testing of different blocking α-CD21 antibodies (data not shown). However, the question whether integrins are involved in EBV infection was not solved. Conclusively, we considered the possibility that the commercially available inhibitory antibody was not hindering EBV binding to the B cell.

Based on these observations, we performed silencing of α4 integrin in Raji cells by siRNA. An efficient silencing in Raji cells with siRNA specific for α4 integrin could not be

achieved although four siRNA sequences were tested. Therefore, as expected, EBV infection of cells treated with siRNA specific for α4 integrin resulted in no difference in susceptibility to EBV infection in comparison to wildtype cells. In contrast, silencing with specific siRNA for GAPDH showed a reduction of the mRNA level of more than 90%, indicating the correct technique was applied for siRNA delivery into Raji cells. Most probably, the sequences are not effective in target silencing. In addition, gene knockdown with shRNA encoding α4 integrin or CD21 was performed. Although five shRNA sequences were tested, there was no decrease of protein expression. This was surprising, because lentiviral transduction is usually very efficient. An improvement of silencing could probably be achieved with a modified protocol. A last try to silence α4 integrin could be conducted with plasmids encoding α4 integrin shRNA, since transfection of B cells is now well established in our laboratory.

Separation of naïve and memory B cells from tonsils by MACS

It would be an advantage to test the findings of this dissertation in primary B cells. This includes several experiments, such as RT-PCR analysis and overexpression studies. For these experiments, it would be very convenient to have separated naïve and memory B cells to investigate the subpopulations in terms of different susceptibilities to EBV infection. This can be achieved for example by separation by MACS. This technique was developed for separation of naïve and memory B cells from the peripheral blood and follows a two-step protocol. First, all B cells ($CD19^+$) are separated by a negative selection. In a second step memory B cells ($CD19^+CD27^+$) are separated from the naïve B cells ($CD19^+CD27^-$) by a positive labeling of the CD27 marker. The separation can finally be verified by flow cytometry analysis for purity.

We followed this protocol to separate naïve and memory B cells from tonsils, and the first step of $CD19^+$ cell separation was always successful, according to the flow cytometry analysis. The $CD19^+$ population reached usually a purity of up to 96%. In contrast, the positive separation of memory B cells, failed regularly and $CD19^+CD27^+$ cells could not be visualized by flow cytometry. The use of different anti-CD27 antibody clones for flow cytometry could not solve the problem. Finally, a different way of separating naïve and memory B cells was successful. Memory B cells from tonsils could be separated from the naïve B cells by an IgD negative instead of a CD27 positive separation.

In this experimental setup, it might be possible that the MicroBead-coupled anti-CD19 antibody prevents the binding of the fluorochrome-labeled anti-CD27 antibody, which is

normally used in flow cytometry. . However, this explanation does not seem to be very likely, since memory B cells from tonsils and the peripheral blood must have a similar phenotype.

The work presented in this dissertation enhances our understanding of the involvement of integrin in EBV entry into B cells and demonstrates a novel role for α4 integrin in EBV infection of B cells. Additional work is necessary to dissect the role of α4 integrin in other B cells, such as the BL cell line BJAB or the LCL derived from a CD21 negative donor. To gain a deeper insight into these findings, the direct interaction of α4β1 with EBV could be studied by colocalization studies. For this studies an effective staining of α4β1 integrin and EBV is necessary. Additionally, it might be interesting to investigate which EBV molecule might be involved in tethering of EBV to the B cells while interacting with cellular α4 integrin. Investigations of EBV entry receptors, potentially responsible for the entry of EBV into epithelial cells, revealed two receptors. In this context, the viral BMRF2 interacts with α3, α5 and β1 integrin (74), whereas the receptor gHgL interacts with αvβ5, αvβ6 and αvβ8 (27).

Investigating the protein sequence of EBV, I found that the viral glycoprotein gp350 contains a LDV motiv. This acidic peptide motiv was shown to be critical for the mechanism of ligand recognition by α4 integrin. Conducting experiments using a recombinant EBV containing no gp350 could exhibit whether gp350 plays a role in facilitating EBV entry into B cells. And finally, a co-immunoprecipitation could further elucidate existing protein-protein interactions.

While the present study has focused on the analysis of alternative B cell receptors involved in EBV entry into B cells, future studies will be necessary to investigate the impact of these receptors on B cells upon EBV binding and determined the unknown binding partner for α4 integrin on the side of EBV.

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Manuscripts

1) The role of α4 integrin in EBV entry

Patricia Krukowski, Florian Haas, Michele Bernasconi and David Nadal

Manuscript in preparation

2) Early gene expression changes by Epstein-Barr virus infection of B cells indicate CDKs and survivin as therapeutic targets for post-transplant lymphoproliferative diseases

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Published in International Journal of Cancer, 2013

1) The role of α4 integrin in EBV entry into B cells

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The γ-herpesvirus Epstein Barr virus (EBV) was demonstrated to infect B cells in a CD21-dependent and -independent manner. We have previously shown that α5β1 plays a role in EBV infection of B cells however the role of other integrins in this process remains elusive. We investigated Burkitt lymphoma (BL) cell lines and primary B cells from tonsils (TBCs) for their integrin expression. We show here that the BL cell line Raji expresses α4 integrin more abundantly than TBCs and is also more susceptible to EBV infection than the latter. Moreover, overexpression of the subunit α4 integrin facilitated EBV entry into the EBV-negative cell line Akata31, but not in the EBV negative cell line BJAB. Our data indicate that α4β1 integrin plays a role in the process of B cell infection by EBV and may contribute to enhanced susceptibility of B cell subsets to EBV infection thus promoting survival of the virus.

Introduction

More than 95% of the human adult population is infected with the gammaherpesvirus Epstein-Barr virus (EBV) (24). Upon infection EBV remains life long in a B cell pool since the EBV-specific immune response controls primary infection but cannot expulse the virus from the body (16, 17).

EBV is associated with several human cancers including B cell lymphoma and nasopharyngeal carcinoma. Moreover, EBV is capable to transform B cells in vitro (1).

EBV can infect both epithelial cells and B cells but the preferential target cells are B cells. The main factors involved in EBV entry into B cells were identified: The viral glycoprotein gp350/220 is responsible for EBV attachment to the complement receptor 2 (CR2), the cellular CD21 (11, 35). Fusion of EBV with B cells requires the viral glycoprotein gB and the tripeptide gHgLgp42. gp42 interacts with HLA class II and triggers fusion of EBV envelope and B cell membrane (2, 14, 15, 22, 39).

Recently, the complement receptor 1 (CD35) was shown to be an attachment receptor for EBV on B cells as well but this was restricted to primary B cells and was not found on B-LCLs and most Tumor cell lines (27).

Although EBV is known to infect B cells without the expression of CD21 (36) little is known about the contribution of other cellular receptors to viral attachment or binding on B cells. In epithelial cells, which do not express CD21 and HLA class II, integrins were used as triggers for EBV fusion. Integrins belong to a large family of transmembrane cell surface receptors consisting of a non-covalently linked α and β subunit (31). They serve as adhesion receptors and are important for contact to their environment as well as for cell-to-cell communication and are therefore attractive candidates for viral attachment (34). The integrins αVβ1, α3β1 and α5β1 were found to be receptors for EBV on oral epithelial cells (41).

We previously showed that β1 integrin is involved in conferring a higher susceptibility to EBV on memory B cells from tonsils (9). But most probably other integrins were also involved in EBV entry into B cells. Several integrins are known to be expressed on B cells. αMβ2 was shown to be involved in B cell adhesion to the basolateral membrane of epithelial cells during transfer infection of EBV (30). Integrin α6β1 and αVβ1 were exhibited to be important during B cell activation of tonsillar B cells (28). αV was demonstrated to be expressed at very low levels on primary B cells (18). Additionally, αLβ2, α4β1 and α4β7 are crucial for migration and development of B cells (43). Because B cells express a variety of integrins, it is of interest to determine their distinct implication in EBV attachment on B cells.

Our findings demonstrate that α4 integrin, which is expressed in most B cell lines facilitates EBV entry into the EBV negative cell line Akata31 but not in the EBV negative cell line BJAB. Our data indicate that α4 integrin together with β1 plays a role as receptor for EBV entry into host cells when CD21 is absent but most possibly also to tether EBV closer to the B cell membrane.

Results

Susceptibility of autologous tonsillar and peripheral blood B cells to EBV infection

In our previous study (10) we compared the susceptibility of naïve and memory B cells from tonsil (TBCs) to EBV infection in vitro with that of their counterparts in peripheral blood (PBBCs). Nevertheless, we did not compare the susceptibility of TBCs and PBBCs to EBV infection from autologous donors and we used a recombinant EBV strain with a latenet membrane protein 2 (LMP2) deletion after insertion of the green fluorescence protein (GFP) gene (32, 33). Therefore, we investigated whether autologous TBCs and PBBCs follow the same pattern of infection after exposure to a BAC-recombinant EBV wt in vitro as in the prior study. EBV wt has the advantage of being derived from wild type EBV and has no gene deletion (7).

To this end, autologous TBCs and PBBCs from three EBV-seronegative and two EBV-seropositive individuals were spinoculated for one hour with EBV (MOI=1.8). 72 hours post inoculation cells were stained with antibodies against CD19 and CD27 and naïve ($CD19^+CD27^-$) and memory ($CD19^+CD27^+$) B cells expressing GFP were identified by flow cytometry. We found that memory TBCs were similarly often infected with EBV as their naïve counterparts (3.9% ±2.6 vs.1.29%±1.1; $P > 0.05$; Fig. 1). In peripheral blood we found also no difference in frequency of EBV infection between naïve and memory B cells (5.1% ±2.0 vs. 5.3% ±2.4; Fig. 1). These results suggest a similar susceptibility to EBV infection for naïve and memory B cells from autologous tonsils and peripheral blood, respectively. This finding stands in contrast t o our previous report (10), where EBV with a LMP2 deletion was used. In our previous study memory B cells from peripheral blood were less susceptible to EBV infection compared to their naive counterparts whereas memory and naïve B cells from tonsil were similarly susceptible to EBV infection (9). Thus, the present study demonstrated that memory and naïve B cells from TBCs and PBBCs are equally susceptible to EBV when using autologous samples and an EBV strain without LMP2 deletion.

Establishment of a primary B cell-independent *in vitro* model to study EBV infection of B cells

Above-mentioned experiments revealed a rather remarkable variation of the B cell susceptibility to EBV infection between donors, as reflected by the rather large standard deviation of the frequencies of EBV-infected cells after exposure to EBV (Fig. 1). This suggests that for further investigation of potential additional receptors for EBV a more uniform model of infection would be preferable. We reasoned that using a B cell line to model cellular EBV infection might ensure more uniform and robust results. Thus, we embarked in searching for a B cell line, which exhibits similar expression of the main entry receptors for EBV as TBCs, i.e., CD21 and HLA-DR.

To this end, we characterized BL cell lines including Raji, Daudi, BJAB, Ramos, Akata31, Mutu I, BL2, Akata, the lymphoblastoid cell line L2, and TBCs for the expression of CD21 and HLA-DR and in addition of the recently reported EBV receptor CD35 (27) by flow cytometry. All BL cell lines expressed CD21 and HLA-DR (Table 1). The percentages of CD21 positive cells were similarly high in Raji (99.1%), Daudi (94.7%), Ramos (91.4%), L2 (88.7%) and Akata Takada (98.2%) in comparison to TBCs (98.3%), whereas less BJAB cells (75.6%) and only a minority of Akata31 cells (5.4%) were CD21 positive. The BL cell line Raji showed the highest Mean Fluorescence Intensity (MFI) of CD21. The percentages of HLA-DR positive cells were in all BL cell lines and TBCs equally high. Only TBCs were positive for CD35, which was also confirmed by Ogembo et al. (27). These data suggest that CD21 is expressed on all BL cells at various levels and HLA-DR is expressed at uniformly high levels.

Integrins may possibly function as receptors contributing to EBV infection susceptibility (5, 9, 37). To first have an overview of integrins expressed in B cells, we characterized the expression of α and β integrin subunits at the RNA level by real-time PCR in TBCs and the BL cell lines Raji, Daudi, BJAB, Ramos, Akata31, MutuI, BL2, Akata Takada, and the lymphoblastoid cell line L2 (supplementary 1 and Fig. 2). Seven alpha (Fig. 2A) and four β integrin subunits (Fig. 2B) were analyzed for their presence in TBCs, Raji, BJAB and Akata31 (αL, αM, αV, α3, α4, α5, α6, β1, β2, β7). α3 integrin is known to be expressed by epithelial and endothelial cells (40). Integrins exist as noncovalently linked α and β subunits which pair to heterodimers and on B cells they associate as αVβ1, α5β1, α4β1, α6β1, αLβ2 and αMβ2.

In the following experiments BL cell lines with differential expression of CD21 (Table 1) that were compared to TBCs were selected.

TBCs were separated from mononuclear cells by negative selection using micro beads (MACS). The purity of the separated $CD19^+$ cells was > 96%, as determined by flow cytometry (data not shown).

In Figure 2 we compared the expression of integrins in TBCs to that in Raji, BJAB and Akata31. We found that two integrins αLβ2 and α4β1 were highly expressed in TBCs and to a lower extent also in Raji, BJAB and Akata31 (Fig. 2). α5β1 and αMβ2 integrin were expressed at a very low level and only in TBCs. Integrins α3β1 and α6β4 were detected at very low mRNA levels in TBCs and the three BL cell lines.

In summary, TBCs expressed the highest levels of integrins and predominantly αLβ2 and α4β1.

To validate the obtained mRNA data we analyzed the integrin expression at protein level by flow cytometry. Integrins, which were hardly detectable at mRNA level and thus assumed to have no expression on B cells like β4, α3 and αE integrin were excluded. TBCs, Raji, BJAB and Akata31 cells were stained with antibodies against integrins αL, αV, α4, α5, α6, β1, β2 and β7 detected by flow cytometry (Fig. 3).

TBCs expressed all examined integrins, whereas BL cell lines displayed integrins in varying degrees (Table 2). β1 and α6 integrin were expressed by all cell lines and TBCs (>65% and 17-38% respectively). β2 integrin was expressed by all cell lines and TBCs (67-99%) but Akata31.

TBCs expressed αL and α5 integrin in >12%. BJAB cells were the only non-primary B cells that expressed αL integrin (52%). αV integrin was expressed in TBCs (7%) and BJAB cells (11%). Solely TBCs expressed β7 integrin.

α4β1 was expressed in all cell lines but Akata31. Of note is that the very susceptible cell line Raji expressed high levels of α4β1, whereby this integrin is absent in Akata31.

Susceptibility of B cells to EBV infection correlates with integrin expression pattern

Next, we functionally investigated the susceptibility of BL cells to EBV infection in comparison to TBCs. Thus, cells were inoculated with GFP-tagged EBV (MOI = 8), and 72 hours post inoculation cells were analyzed by flow cytometry for GFP expression. 6% of TBCs, 18% of BJAB, 24% of Raji and 3% of Akata31 cells were positive for GFP (Fig. 4).

Hence, Raji cells were the cells most susceptible to EBV infection, followed by BJAB, TBCs and Akata31 cells. Thus, given that Raji cells expressed very frequently α4 integrin (97%), which was also expressed in BJAB cells or TBCs, we hypothesized a possible role for α4 integrin in EBV entry into B cells.

Complexation of divalent cations reduces susceptibility to EBV infection

Integrin interactions with extracellular ligands are dependent on divalent cations, including Ca^{2+}, Mn^{2+} and Mg^{2+} (19). The divalent chelator ethylenediaminetetraacetic acid (EDTA) alters affinity in integrin-ligand binding by complexing divalent cations, which has been shown to reduce binding of integrins to viruses (19).

Therefore, we investigated the effect of EDTA on susceptibility of Raji cells to EBV infection. Raji cells were chosen due to their high susceptibility to EBV infection and similar expression of CD21 and HLA-DR as TBCs. This would therefore best allow to screen for the potential role of integrins in enhancing cellular EBV infection. Raji cells were incubated with increasing concentrations of EDTA and subsequently spinoculated with an EBV at a MOI=5. After 72 hours, Raji cells were analysed for GFP expression by flow cytometry. EDTA diminished EBV infection in Raji cells in a dose-dependent manner. The blocking effect of EDTA on EBV infection between control cells and cells treated with the highest concentration was 20% (Fig. 5A).

Earlier we showed that inhibitory antibodies against CD21 and HLA-DR reduced the level of susceptibility to EBV infection of primary B cells up to 80% (9). A complete inhibition, however, was not achieved, suggesting additional receptors might be involved in EBV entry into B cells. The 20% of EDTA blocking effect would fill the gap and complete the missing unknown receptor. We next sought to determine whether additional incubation with EDTA could further reduce the already decreased level of susceptibility to EBV infection by inhibitory antibodies. Therefore Raji cells were incubated with EDTA or EDTA and inhibitory antibodies against CD21 and HLA-DR or both. After 72 hours, cells were preceded to flow cytometry analysis for GFP expression. Application of inhibitory antibodies alone reduced the susceptibility to EBV infection of about 42% (α-HLA-DR), 55% (α-CD21) or 80% when combining both antibodies, whereas the additional incubation with EDTA did not exhibit any further reduction of EBV susceptibility to EBV infection. Since incubation of antibodies and EDTA from Figure 5B resulted in inappropriate conditions for EDTA, the perfect conditions must be further established.

Thus, we could show that EDTA exerts an inhibitory effect on EBV infection, which emphasizes the potential involvement of integrins in EBV infection of B cells.

Effect of reduced expression of α4 integrin on susceptibility to EBV infection

To determine whether reduced expression of α4 integrin reduces B cell susceptibility to EBV infection, we established α4 integrin silencing in Raji cells by use of siRNA. Transfection of Raji cells with the fluorochrome-tagged control siRNA showed a transfection efficiency of 93% in comparison to non-transfected cells (not shown). To test the efficiency for siRNA-based silencing in Raji cells we employed first a validated siRNA for GAPDH. Transfection with the GAPDH-specific siRNA reduced mRNA level of 85 % in comparison to non-specific siRNA (not shown). Transfection of siRNA against α4 integrin using one distinct single sequence (S1) or the pool of four sequences reduced mRNA levels by about 75% and 50%, respectively (Fig. 6A).

To further examine the modest silencing efficiency, transfected Raji cells were stained for α4 integrin at the cell surface and expression was analysed by flow cytometry. Use of α4-specific siRNA reduced protein expression by 10% or 25% 24 hours or 48 hours post transfection, respectively (Fig. 6B).

To determine whether the limited reduction of α4 integrin expression would affect susceptibility to EBV infection, we transfected Raji cells with siRNA specific for the sequence S1 against α4 integrin.

48 hours post transfection Raji cells were spinoculated with EBV (MOI = 8) and incubated for another 48 hours. Thereafter, cells were analyzed for GFP expression by flow cytometry (Fig. 6C). The α4 integrin-specific siRNA did not exhibit any inhibitory effect on EBV infection as compared to non-specific siRNA (Fig. 6C). We speculate that the silencing effect of siRNA was not sufficient enough to reduce cell surface expression of α4 integrin below a critical level to reduce the efficiency of EBV infection.

Overexpression of α4 integrin in Akata31 cells results in higher susceptibility to infection with EBV

We showed that Akata31 cells are poorly susceptible to EBV infection. Their expression of CD21 and α4 integrin is very low. Thus, the potential role of α4 integrin in EBV infection can be examined by overexpression of the integrin in Akata31, especially considering the absence of CD21 in this cell line.

To determine whether gain of function of α4 integrin alters the susceptibility to EBV infection in Akata31 or in the more susceptible BJAB cells, we transfected an expression plasmid encoding α4 integrin and empty vector. Wt cells and empty vector transfected cells were used as controls. As determined by Western blot analysis, α4 integrin expression was highly increased in empty vector cells or parental cell line (Fig. 7A). To verify that the expression level of other integrins on the cell surface was not altered by overexpression of α4 integrin we analyzed the expression of key integrins by flow cytometry.

The percentage of expression and the mean fluorescence intensity (MFI) of all investigated integrins were not altered except of α4 and β1 integrin in Akata31 cells (Fig. 7B) and BJAB cells (Fig. 7C). In Akata31 cells overexpressing α4 integrin, the percentage of cells expressing α4 integrin increased from 0 up to 100% and in BJAB from 10 to 80%. The expression level and intensity of CD21 and HLA-DR on Akata31 (Fig. 7D) and BJAB (Fig. 7E) was not affected. Hence overexpression of α4 increased surface expression of α4β1 but not any other investigated receptor.

To determine whether increased α4β1 integrin expression alters the susceptibility of Akata31 or BJAB cells to EBV infection, cells were incubated with the GFP recombinant EBV.

Figure 8A and B show dot plots depicting the expression of α4 integrin in wt, empty vector control (EV) and stable transfected cell lines (+α4) in Akata31 and BJAB. Expression of α4 integrin in Akata31 cells increased their susceptibility to EBV infection by two-fold whereas in BJAB cells no such an effect was observed (Fig. 8C and D). This data suggests that α4 integrin over expression on CD21-negative cells promotes EBV attachment on B cells. The data further suggest that α4β1 integrin inherits partially the role of CD21 in the CD21-negative cell line Akata31.

Discussion

In this study we aimed at investigating the potential role of integrins other than α5β1 integrin in enhancing the susceptibility of B cells to infection with EBV. We show that (i) Raji cells which express more α4 integrin than primary B cells from tonsils are more susceptible to EBV infection than the latter; (ii) treatment of Raji cells with EDTA that inhibits the action of integrins reduced their susceptibility to EBV infection; and (iii) Akata31 cells which lack α4 integrin were less susceptible to EBV infection than primary tonsillar B cells but were rendered more susceptible to EBV infection by overexpression of α4 integrin. Our observations suggest that α4 integrin is involved in the process of B cell infection by EBV and may contribute to enhancing susceptibility of B cell subsets that may promote survival of the virus.

An intriguing observation in our study was that Raji cells were more susceptible to EBV infection in vitro than primary B cells. Given that Raji cells and primary B cells express similar levels of the main known receptors for EBV, i.e. CD21 and HLA-DR, we hypothesized that the increased susceptibility of Raji cells can be attributed to their markedly higher expression of α4 integrin compared to primary B cells. Notably, recent studies on EBV entry revealed that, depending on the cell type, different integrins are involved in viral host cell entry. In polarized epithelial cells α3, α5, αV (41) and β1 integrin (37, 41) have been shown to play a role in EBV infection, whereas in B cells only α5β1 (9) was shown to be involved. The difference between Raji cells and TBCs is unlikely due to an even more abundant expression of the recently described EBV receptor in B cell, i.e. CD35, since we confirmed that CD35 is absent n Raji cells (27). Thus, absence of CD35 did not seem to reduce susceptibility to EBV infection in comparison to primary B cells that express CD35. Instead, the more marked expression of α4 integrin compared to primary B cells suggested that this integrin might indeed be involved in the B cell infection process.

Our finding that treatment of Raji cells with EDTA resulted in reduced susceptibility of Raji cells to EBV infection further underscores that integrins may indeed be involved in EBV infection of B cells. Nevertheless, treatment of cells with the divalent cation complexer EDTA plus blocking of CD21 and HLA-DR by the use of inhibitory antibodies did not result in a complete inhibition of EBV infection. (This experiment will be repeated, since the experiment was done only once and the ideal protocol was not established yet). However, a possible additive effect of CD21 or HLA-DR blocking antibodies and EDTA to inhibit integrins still has not been investigated in detail. Impossible to establish efficient silencing of

An important observation pointing to the role of α4 integrin in enhancing susceptibility of B cells to EBV infection was that Akata3 cells overexpressing α4 integrin exhibited a two-fold higher susceptibility to EBV infection than parental wild type Akata31 cells. This is especially intriguing since we showed here that Akata31 cells do not express CD21. Thus, α4 integrin does not seem to need collaboration with CD21 but is capable to enhance susceptibility to infection by its own. In this regard α4 integrin likely acts as an attachment receptor, however further investigation is required to determine the contribution of active α4 integrin signaling. A study demonstrated that interaction of α4β1 integrin on germinal centre (GC) B cells with its ligand VCAM-1 could prevent the cells from undergoing apoptosis. This suggested that activation of the α4 integrin pathway may contribute to the B cell selection process (20). The result of induced GC B cells by α4 binding via EBV exhibited an evolutionary advantage to naïve B cells in preventing EBV infected B cells from apoptosis and thus shifts the survival rate in favor of EBV infected cells.

Use of host cell signaling to enter target cells by viruses and therefore establishing a productive infection, was documented for the herpes virus Kaposi sarcoma herpesvirus (KSHV). KSHV activates the FAK, Src, PI3K, Rho-GTPases as well as several other downstream effector molecules, initiating actin rearrangements that lead to the internalization of KSHV (4).

Likewise, EBV was shown to activate integrin signaling and thereby enhances infection efficiency in memory B cells from tonsils. Binding via its glycoprotein BMRF-2 to α5β1 EBV initiates FAK, Scr and PI3K to finally activate the actin cytoskeleton (9).

Furthermore, the access to α4 by EBV might be even promoted by an association of α4β1 integrin with CD81. It was shown that CD81 could associate with α4β1 integrin in cell surface clusters on B cells and promotes cell processes (25). This is also the case for KSHV, where integrins associate with other receptors, such as CD98 and thus may form an entry complex (12, 38). CD98 was also demonstrated to be involved in the post entry stage of KSHV infection, most probably by mediating a signaling cascade that is important for viral-gene expression (38).

Integrins are cell surface receptors, involved in signaling and cell adhesion. Dependent on the associated β subunit, α4 integrin may preferentially bind to the vascular cell adhesion molecule 1 (VCAM-1) or the mucosal vascular addressin cell adhesion molecule 1 (MadCam-1). VCAM-1, which is mainly responsible for the recruitment of α4β1 high expressing cells, contributes to lymphocyte homing to oropharynx and BALT (42) and hence to the portal of

EBV spreading. Thus, EBV positive B cells expressing α4β1 are likely to return to the oropharynx from where the virus can be transmitted via saliva to other recipients.

The results presented here do not define the precise step in EBV cell entry mediated by α4 integrin. EBV could use α4 which we showed associates preferentially with β1 integrin, essentially as an attachment receptor on B cells or as signaling factor after viral attachment, or both. Additionally, the structural EBV capsid protein interacting with α4β1 integrin remains elusive. Further studies aiming at elucidating the direct association of EBV with α4 integrin will provide inside into these questions. A further limitation was found in approaches to diminish expression of α4 integrin subunit in B cells by RNA interference. B cells are highly resistant to conventional transfection methods (e.g. cationic lipids and polymers) (13, 26) but can be transduced in vitro by electroporation. Achievement of reduced α4 integrin expression with specific siRNA against α4 integrin in Raji cells was poor and consequently a reduction of EBV infection was not observed. Since the transfection with electroporation was very efficient (not shown), and half-life of integrin protein in cultured cells (fibroblasts) is about 6-18h (6, 23) we suggest a siRNA design problem to cause an insufficient silencing.

Our results indicate that α4β1 integrin contributes to enhanced susceptibility of B cells to infection with EBV. This may be beneficial for EBV transmission to other hosts, as infected B cells expressing α4β1 integrin are likely to home to the oropharynx and BALT, the portal of exit of EBV.

Acknowledgements:
We thank Mark H. Ginsberg from the University of California for providing the α4 integrin plasmid.

Material and methods

Cell culture and virus.

All B cells were maintained in RPMI 1640 supplemented with 10% fetal bovine serum (FBS) (Invitrogen, Basel, Switzerland), 1% L-glutamine, and 1% penicillin-streptomycin (from Gibco, Basel, Switzerland) in a humidified 5% CO2 atmosphere at 37°C.

Primary B cells derived from tonsil. All B cell lines originate from Burkitt Lymphoma biopsies. The cell lines Raji, Daudi are EBV positive and BJAB, Akata31 and Ramos are EBV negative.

HEK 293 cells carrying stably wildtype EBV BAC (D2089; EBV-wt B95.8)) (kindly provided by Henri Jacques Delecluse (DKFZ, Heidelberg, Germany) have been previously described (7), Cells were maintained in DMEM, 10% fetal bovine serum (FBS), 1% L-glutamine, 1% penicillin-streptomycin and 100 µg/ml Hygromycin (LabForce, Nunningen, Switzerland).

Antibodies and reagents.

Anti- CD19 mAb pacific blue (PB)-LT19 (AbD Serotec, Düsseldorf, Germany), anti-HLA-DR mab APC/Cy7-L243 (Biolegend, San Diego, United States) anti-CD27 mAb phycoerythrin (PE)-L128 (BD Bioscience, Allschwil, Switzerland), anti-integrin α4/CD49d mAb (PE)-9F10, anti-integrin α5/CD49e mAb (PE)-IIA1, anti-integrin β1/CD29 mAb (PE)-MAR4, anti-integrin β2/CD18 mAb (PE)-6.7 (all from BD Bioscience, Allschwil, Switzerland). Anti-integrin β7 mAb (PE)-473207, anti-integrin αV/CD51 mAb (PE)-P2W7, anti-integrin αL/CD11a mAb (PE)-345913 (R&D systems, Abingdon, UK), anti-integrin α6/CD49f mAb (PE)-3H1512 (US Biological, Massachusettes, United States .

Anti-α4 integrin antibody for western blot was purchased from cell signalling (Boston, United States).

Isolation of primary cells.

Human mononuclear cells were separated from tonsils of donors undergoing routine tonsillectomy. Preparation of tonsillar mononuclear cells was done as previously described (21).

Virus preparation and infection.

Free virus in supernatant was achieved from induced 293/EBV-wt cells. 293 cells at 80% confluency were transfected in 100 mm Petri dish with expression plasmids (5 µg each/dish)

encoding the BZLF1 and the BALF4 gene products, using liposome-based reagent Metafectene (Biontex, Martinsried/Planegg, Germany). After 5 hours, the transfection mixture was removed and cells were kept in DMEM supplemented with 10% fetal bovine serum (FBS), 1% L-glutamine, and 1% penicillin-streptomycin. Three days post virus induction, virus supernatant was filtered trough a 0.45 µm pore size filter and stored at 4°C until use.

Determination of infectious EBV particles was done like described in (8).

B cells were inoculated or spin inoculated. B cells were then maintained in supplemented RPMI.

For spinoculation, cell suspension was centrifuged for 1 hour at 800 x *g* at 4°C. Cells were subsequently washed in phosphate-buffered saline and resuspended in fresh complete medium.

For concentration of virus filtrated viral supernatant was centrifuged at 30'000 x g for 2 hours at 4°C and than resuspended in 1x PBS and stored at 4°C until further use.

Blocking experiments:

- with EDTA: 2 x 10^5 Raji were used. Dilutions of EDTA were prepared in serum free RPMI (+ Glutamine and + Pen/Strep.) (50mM, 25mM, 12mM, 5mM, 2.5mM, 1.2mM, 0.6mM, 0mM). EDTA-dilutions or medium alone was added to wells in triplicate. Cells were incubated at 37°C for 90 min, followed by incubation at 4°C for 30 min.

EBV-wt (MOI=8) was added to the cells, and were spinoculated (800g x for 60 min at 4°C). Immediately thereafter medium was replaced and cells were incubated for 72 hours at 37°C. Cells were washed and analyzed by flow cytometry.

- with inhibitory antibodies and EDTA: To 2 x 10^5 Raji cells 10 µg/ml of α-CD21 and or α-HLA-DR antibody and 25 mM EDTA was added and incubated for 1h at 4°C. followed by incubation at 4°C for 30 min. EBV-wt (MOI=8) was added to the cells, and were spinoculated (800g x for 60min at 4°C). Immediately thereafter medium was replaced and cells were incubated for 72 hours at 37°C. Cells were washed and analyzed by flow cytometry.

Real-time polymerase chain reaction (RT-PCR).

Total RNA isolation, DNAse treatment, reverse transcription and RT-PCR were performed as described earlier (21, 29).

qPCR was performed for the subunits α3, α4, α5, α6, αE, αL, αM, αV, β1, β2, β4 and β7 and integrin expression was normalized to the expression of the housekeeping gene hydroxymethylbilane-synthase (HMBS) and further normalized to control (depicted as $2^{-\Delta\Delta ct}$). Total RNA was isolated after 24 hours post splitting the cells.
All subunits and HMBS were analyzed using primer/probe on demand (Assay-on-demand gene expression product; Life Technologies Europe B.V., Zug, Switzerland).

Plasmid integration.
Transfections were performed with electroporation Neon system (Life Technologies Europe B.V., Zug, Switzerland) according to the manufacturer's instructions.
24 hours prior to transfection, cells were splitted.
Akata31 (1150-20-2) or BJAB cells (1350-20-1) (pulse voltage-pulse width-pulse number) were transfected with 2.5 μg of α4 integrin plasmid and control pcDNA3.1- (no insert). After 48 hours, cell culture media was exchanged with media containing 800 mg/ml G418 sulfate.
Four weeks later, cells were stained with corresponding integrin antibody and integrin surface expression was determined by flow cytometry.
Anti-Phycoerythrin (PE) Microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) were used for positive selection of α4 integrin positive labelled (PE) cells according to manufacturer's instructions.

Flow cytometry.
Flow cytometry was performed using a FACS Canto II flow cytometer (Beckman Coulter, Nyon, Switzerland). Flow cytometry data were analysed by FlowJo.

Western blot analysis.
Cells (1 x 10^6/ ml) were lysed in lysis buffer (RIPA). Lysates were frozen at -20°C, thawed and centrifuged at 10'000 rpm for 10 min at 4°C. Supernatant was mixed with Roti load (Carl Roth, Karlsruhe, Germany) and boiled at 96°C for 5 min. Proteins were separated by SDS-PAGE and transferred to a nitrocellulose membrane (Whatman, GE Healthcare Europe GmbH, Switzerland) and visualized using specific antibody and ECL (GE Healthcare Europe GmbH, Switzerland).

Knockdown of α4 integrin expression with siRNA.

Raji cells were splitted 24 hours before transfection. At day of transfection, Raji cells were mock transfected or transfected with α4 integrin specific or control siRNA (50 mM)(Qiagen Allstars Negative Control siRNA) using Neon transfection systems (Life Technologies, Zug, Switzerland). After 48 hours of transfection, cells were harvested for detection of surface integrin expression by flow cytometry.

For analysis of infection efficiency, siRNA transfected cells were spinoculated with recombinant EBV wt 48 hours post transfection. After 48 hours cells were harvested and infection rate was determined by flow cytometry.

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Tables and Figures

Table 1

Cells	CD21 [%]	CD21 [MFI]	HLA-DR [%]	HLA-DR [MFI]	CD35 [%]	CD35 [MFI]
TBCs	98.3±1.4	16'935±1'242	98.4±0.2	30'705±2'770	19±10	1447± 281
Raji	99.1±0.3	57'434±7'907	95.2	48'805±5'632	n.d.	n.d.
Daudi	94.7±0.4	5'416±623	99.4±0.3	43'891±5'990	n.d.	n.d.
BJAB	75.6±2.6	2'147±223	99.7±0.2	27'022±3'201	n.d.	n.d.
Ramos	91.4±1.8	4'689±518	98.89±0.2	14'212±2176	n.d.	n.d.
Akata31	5.3±1.2	2'691±303	97.4±0.5	85'746±10'427	n.d.	n.d.
Mutu I	85±1.2	4'856±489	99.3±0.3	54'420±7'702	n.d.	n.d.
BL2	96	1'149±98	97.4±0.5	44'789±7'517	n.d.	n.d.
L2	88.7±1.8	8'744±1'593	98.6±0.5	54'290±9'257	n.d.	n.d.
Akata	98.2±1.3	5'867±781	99.1±0.2	40'278±6'257	n.d.	n.d.

Table 1. Presence of main receptors for EBV entry on various B cells.

TBCs, Raji, BJAB, Ramos, Akata, Akata31, Mutu I, BL2 and L2 were specifically labelled with monoclonal antibodies against CD21, HLA-DR and CD35.

The percentage of stained cells was quantified by flow cytometry. Shown are the percentages of positive cells and the numbers in the right column are the corresponding mean fluorescence intensity (MFI).

Table 2

Integrin	TBCs		Raji		BJAB		Akata31	
αL	18% ± 5	4347 ±3940	n.d.		52% ±4	2638 ±270	n.d.	
αV	7% ± 5	2320 ±1341	n.d.		11% ±4	1157 ±434	n.d.	
α4	49% ±15	8372 ±7254	98% ±2	12197 ±3567	42% ±16	1419 ±950	n.d.	
α5	12% ± 9	480 ±286	n.d.		n.d.		12% ±1	791 ±306
α6	38% ±15	1825 ±621	30% ±22	1968 ±618	33% ±4	1423 ±2005	17% ±4	1063 ±677
β1	65% ±12	6462 ±5626	100% ±1	9041 ±3187	98% ±3	5206 ±2150	76% ±14	1676 ±1169
β2	99% ±1	2642 ±865	67% ±42	2236 ±1410	96% ±2	6137 ±2462	n.d.	
β7	9% ±4	830 ±648	n.d.		n.d.		n.d.	

Table 2. Integrin protein expression of TBCs, Raji, BJAB and Akata31.

TBCs, Raji, BJAB and Akata31 were specifically labelled with monoclonal antibodies against the integrin subunits αL, αv, α4, α5, α6, β1, β2 and β7. The percentage of stained cells was quantified by flow cytometry. Numbers are the percentage of positive cells and the number on the right is the corresponding MFI. The results derived from three independent experiments. n.d. means not detected.

Figure 1

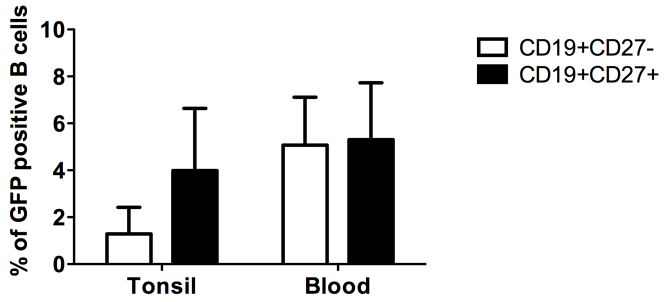

Figure 1. *In vitro* infection of autologous tonsillar and peripheral blood B cells.
Mononuclear cells were harvested from tonsils and isolated by Ficoll-Hypaque density gradient centrifugation. Subsequently the cells were spinoculated with EBV, MOI=1.8. After 72 hours, cells were stained for CD19 and CD27 and analyzed by flow cytometry. The bars represent the naïve ($CD19^+CD27^-$) and memory ($CD19^+CD27^+$) B cells positive for GFP. Data derives from five autologous donors as means ± standard deviation (SD).

Figure 2

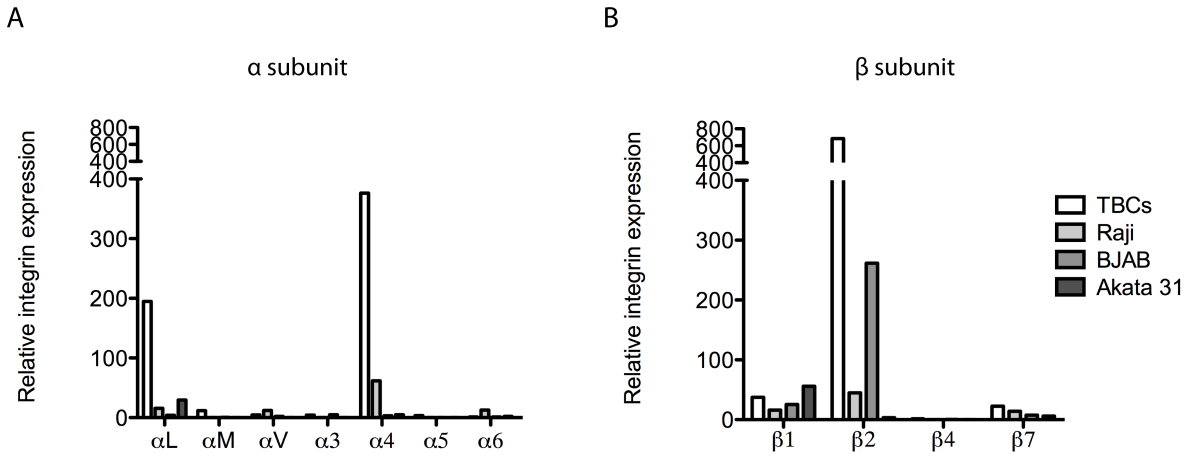

Figure 2. Comparison by quantitative real-time PCR of integrin mRNA expression in primary B cells, Raji, BJAB and Akata31 cells.

Expression level of selected integrins was determined using real-time PCR. mRNA expression is shown of seven α (A) and four β subunits (B). The real-time PCR data was expressed as mRNA levels relative to hydroxymethylbilane synthase (HMBS). Depicted are values as $2^{-\Delta ct}$.

Figure 3

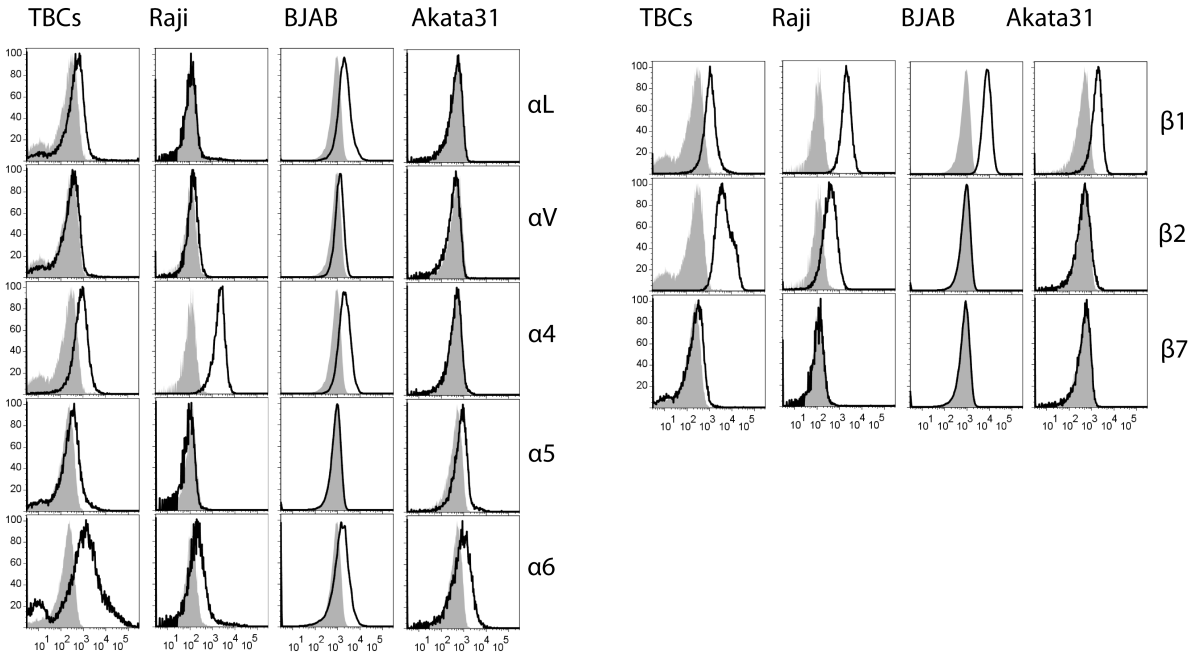

Figure 3. Integrin protein expression of TBCs, Raji, BJAB and Akata31.

TBCs, Raji, BJAB and Akata31 were specifically labelled with monoclonal antibodies against the integrin subunits αL, αv, α4, α5, α6, β1, β2 and β7. Grey shadow represents isotype control and solid line the integrin expression.

Figure 4

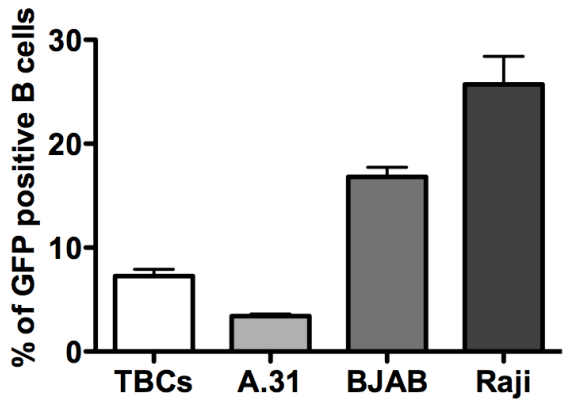

Figure 4. Infection frequency of TBCs, Raji and Akata31.

Freshly isolated TBCs, Akata31, Raji and BJAB were inoculated with EBV (MOI=8) and 72 hours post inoculation cells were analysed for GFP by flow cytometry. The graph represents the percentages of GFP positive cells.

Figure 5

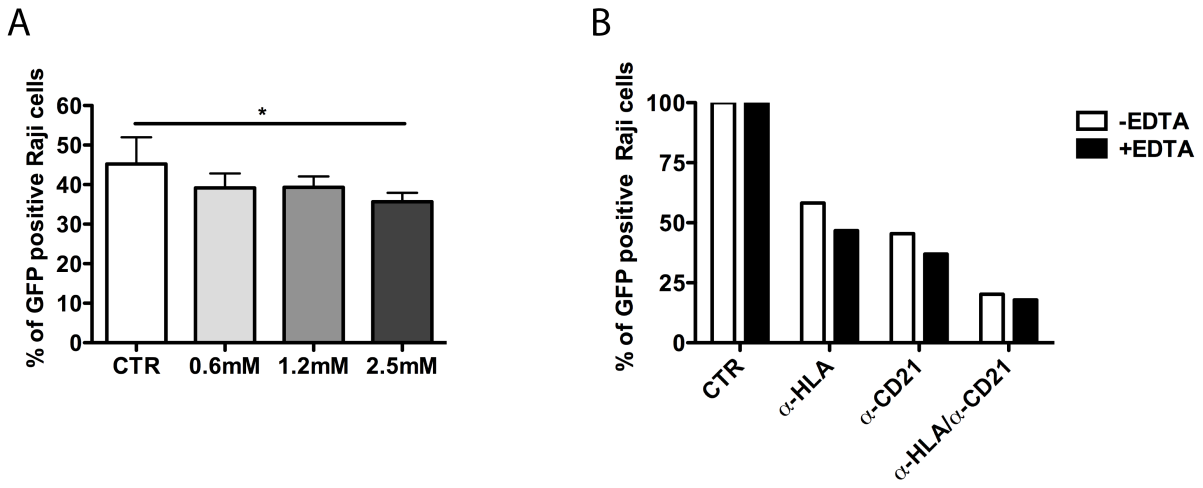

Figure 5. EDTA decreases EBV infection in a dose dependent manner.

(A) Raji cells and EBV were incubated with different concentration of EDTA (0.6 mM, 1.2 mM and 2.5 mM) for 90 min at 37°C following incubation at 4°C for 30 minutes. EBV (MOI=5) was spinoculated with the cells. Infected cells were identified by flow cytometry. CTR means EDTA untreated cells. Results are represented as means ± SD.

(B) Raji cells were incubated with antibodies against CD21 (10 µg/ml), HLA-DR (10 µg/ml), or both prior EBV infection and subsequently EDTA was added (2.5 mM). Infected cells were identified by flow cytometry. CTR cells were calculated as 100%. White bars: addition of blocking antibodies; black bars: addition of EDTA and blocking antibodies.

Figure 6

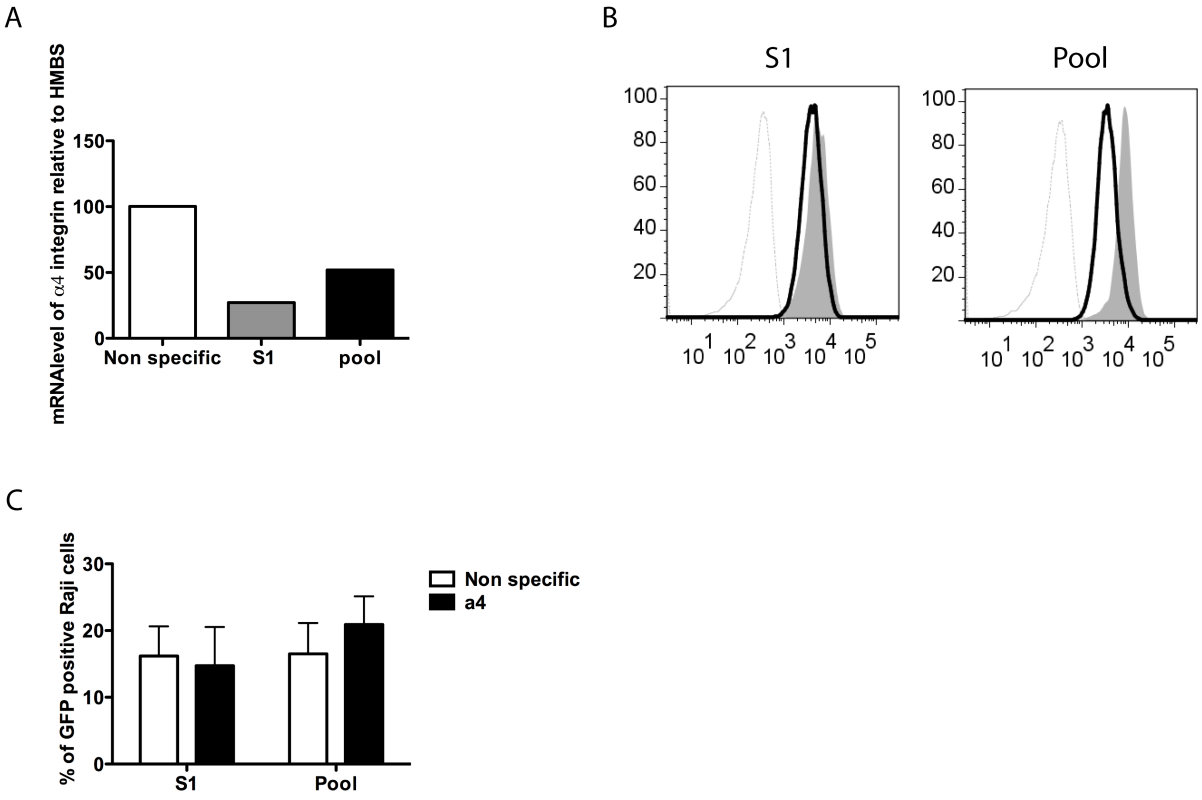

Figure 6. Diminished expression of α4 integrin with siRNA does not decrease EBV susceptibility of Raji cells.

(A) Raji cells were transfected with non-specific control siRNA or either with distinct single sequence siRNA (S1) for α4 integrin or a pool of four sequences. Results are expressed as mRNA levels relative to HMBS 48 hours after transfection. (B) Raji cells transfected with either distinct siRNA (black heavy line) for S1 (left) and pool (right) or with non-specific control siRNA (grey shadow) were stained with antibody against α4 integrin. Transfected cells were identified by flow cytometry for α4 integrin after 48 hours. Dotted line represents isotype control.

(C) Raji cells were transfected with non-specific control siRNA or with distinct siRNA against α4 integrin. After 48 hours cells were spinoculated with EBV. Flow cytometry analysis for GFP was done after additional 48 hours.

Figure 7

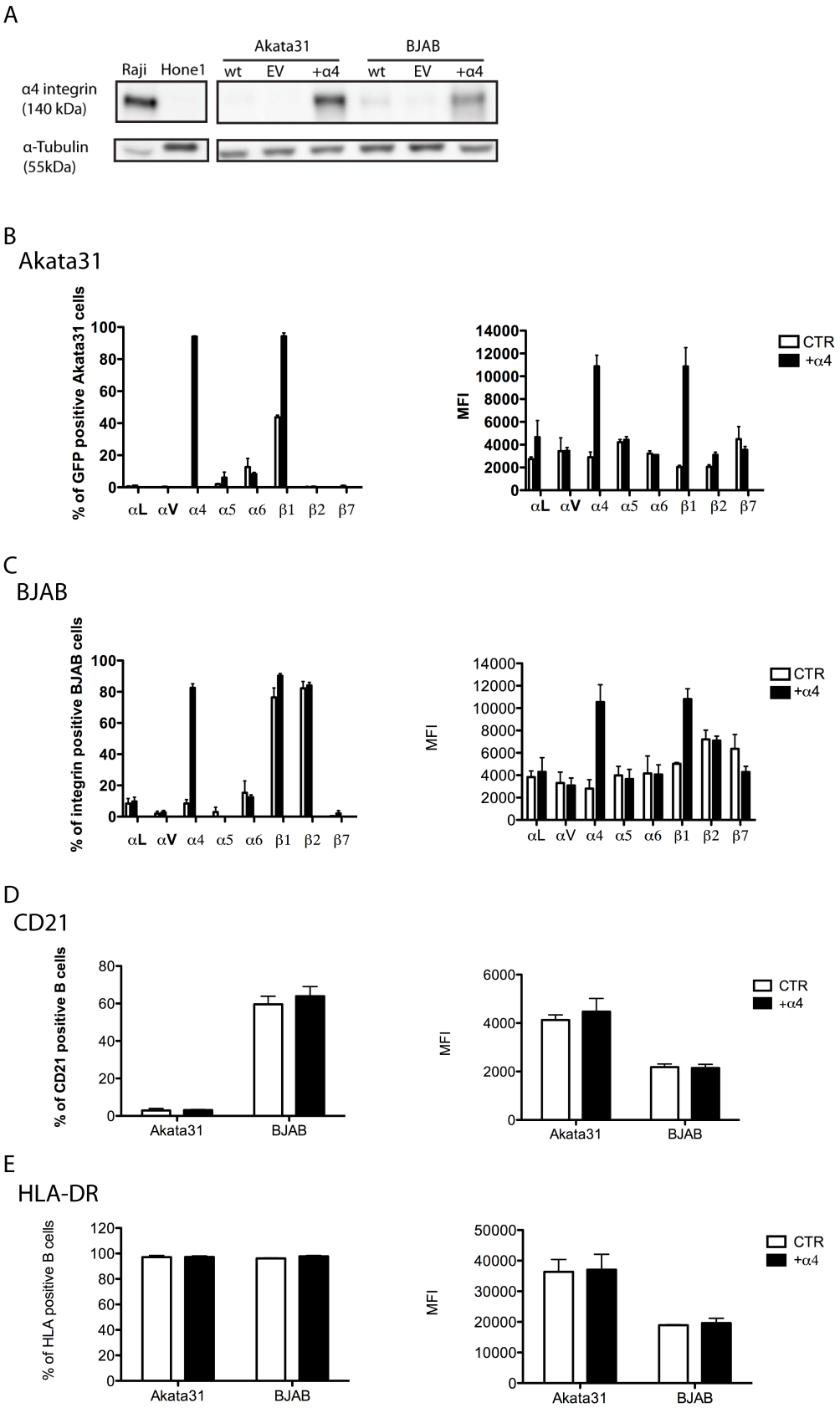
A
Akata31
BJAB
Raji
Hone1
wt
EV
+α4
wt
EV
+α4
α4 integrin
(140 kDa)
α-Tubulin
(55kDa)
B
Akata31
% of GFP positive Akata31 cells
MFI
CTR
+α4
αL αV α4 α5 α6 β1 β2 β7
C
BJAB
% of integrin positive BJAB cells
MFI
CTR
+α4
αL αV α4 α5 α6 β1 β2 β7
D
CD21
% of CD21 positive B cells
MFI
CTR
+α4
Akata31
BJAB
E
HLA-DR
% of HLA positive B cells
MFI
CTR
+α4
Akata31
BJAB

Figure 7. Over expression of α4 integrin in Akata31 and BJAB.

Akata31 and BJAB cells were transfected with a plasmid encoding α4 integrin or empty vector. (A) Protein from whole Akata31 lysate was separated by SDS-PAGE and analyzed by Western blot with anti-α4 integrin mAb after transfer to the membrane. Data shown are representative of three independent experiments. Cell line Raji served as a positive, Hone1 as a negative control. (B) Akata31 and (C) BJAB cells were incubated with mAb against integrin α4, α5, α6 αL, αV, β1, β2 and β7 and subsequently analysed by flow cytometry. (D) Akata31 and BJAB (E) were incubated with antibodies against CD21 and HLA-DR and subsequently analysed by flow cytometry.

Figure 8

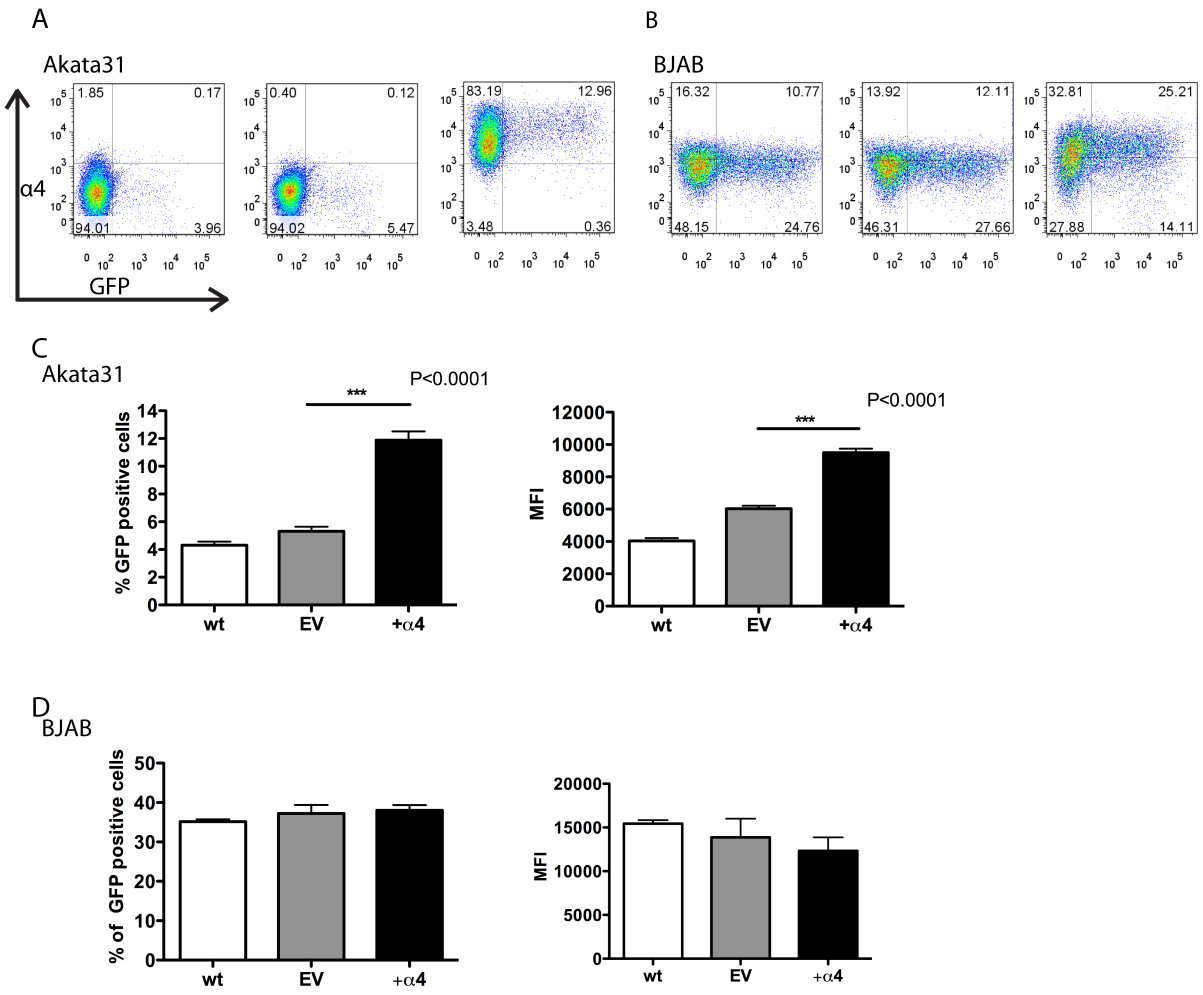

Figure 8. Expression of α4 integrin in Akata31 cells correlates with higher susceptibility to infection with EBV.

Akata31 (Fig. 8A and C) and BJAB (Fig. 8B and D) cells were incubated with the GFP EBV and after 72 hours, cells were analysed by flow cytometry for GFP. Graphs represent the results from A and B in numbers. Results are expressed as the mean percentage of infected cells or the MFI. The results derive from three independent experiments.

Supplementary 1

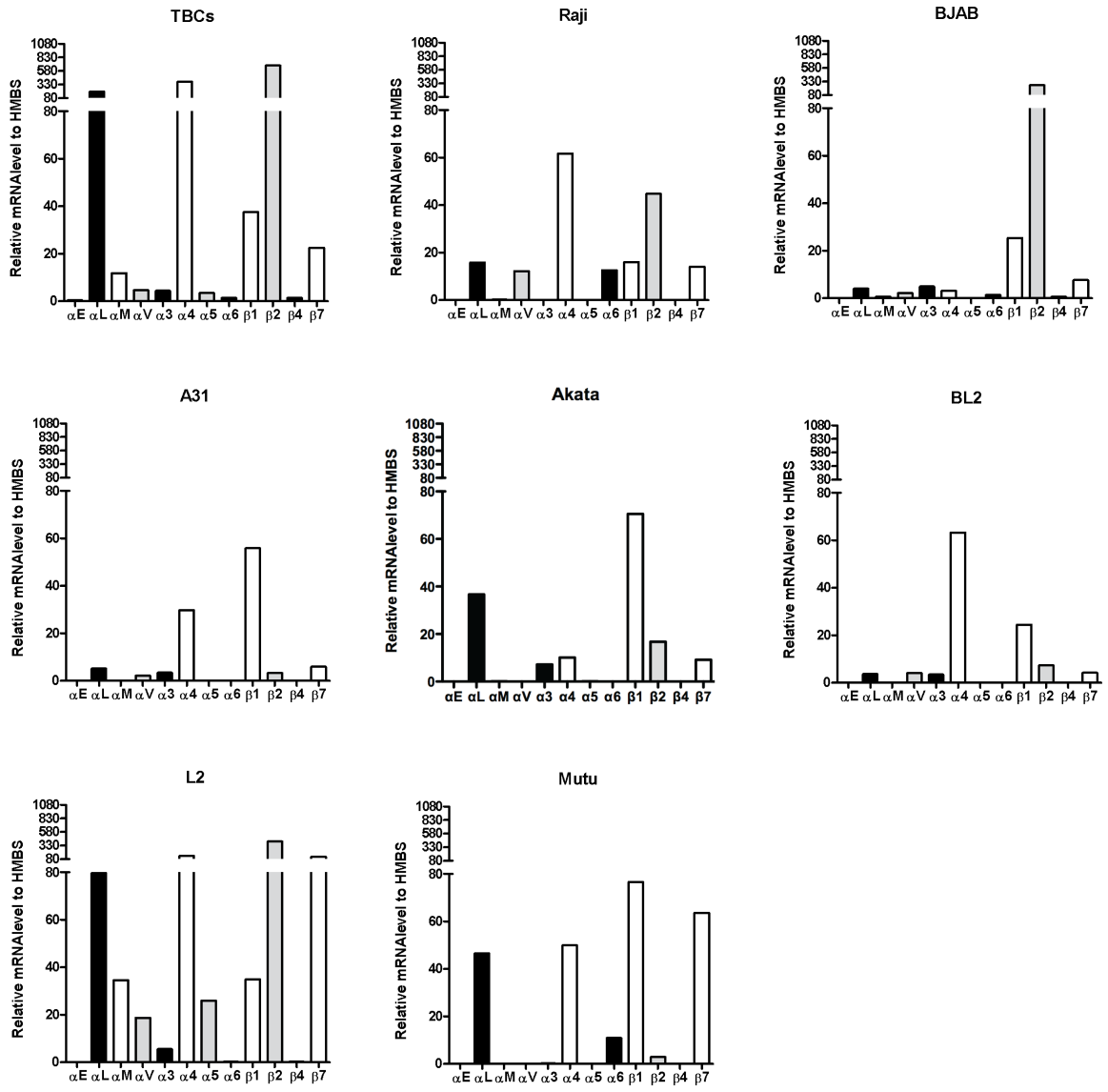

Figure S1. Comparison by quantitative real-time PCR of integrin mRNA expression in TBCs, Raji, BJAB, Akata31, Akata Takada, BL2, L2 and Mutu I cells.

Expression level of selected integrins was determined using real-time PCR. mRNA expression is shown of eight α and three β subunits. The real-time PCR data was expressed as mRNA levels relative to hydroxymethylbilane synthase (HMBS).

Values are depicted as $2^{-\Delta ct}$.

IJC
International Journal of Cancer

Early gene expression changes by Epstein-Barr virus infection of B-cells indicate CDKs and survivin as therapeutic targets for post-transplant lymphoproliferative diseases

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Lymphoproliferative diseases (LPDs) associated with Epstein-Barr virus (EBV) infection cause significant morbidity and mortality in bone marrow and solid organ transplant recipients. To gain insight into LPD pathogenesis and to identify potential effective therapeutic approaches, we investigated early molecular events leading to B-cell transformation by gene expression profiling of EBV-infected B-cells from tonsils by Affymetrix microarray 72 hr postinfection when the B-cells hyperproliferation phase starts. Cell cycle and apoptosis were the most significantly affected pathways and enriched gene sets. In particular, we found significantly increased expression of cyclin-dependent kinase (CDK)1 and CCNB1 (cyclin B1) and of one of their downstream targets BIRC5 (survivin). Importantly, the strong upregulation of the antiapoptotic protein survivin was confirmed in lymphoblastoid cell lines (LCLs) and 71% of EBV-positive post-transplant EBV-LPD lesions scored positive for survivin. The validity of early transforming events for the identification of therapeutic targets for EBV-LPD was confirmed by the marked antiproliferative effect of the CDK inhibitor flavopiridol on LCLs and by the strong induction of apoptosis by survivin inhibition with YM155 or terameprocol. Our results suggest that targeting of CDKs and/or survivin in post-transplant EBV-LPD by specific inhibitors might be an important approach to control and eliminate EBV-transformed B-cells that should be further considered.

Lymphoproliferative disease (LPD) associated with the B-cell tropic Epstein-Barr virus (EBV) causes significant morbidity and mortality in solid organ transplant (SOT)[1] or bone marrow transplant (BMT)[2] recipients. The risk to develop EBV-LPD is greatest in EBV-seronegative transplant recipients, *i.e.*, upon primary EBV infection, and in patients with extensive iatrogenic immunosuppression.[3] EBV-associated B-cell lymphoma in the setting of post-transplant LPD (PTLD) is

Key words: Epstein-Barr virus (EBV), survivin, post-transplant lymphoproliferative disorder (PTLD)
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Grant sponsor: Swiss National Foundation; **Grant numbers:** 310040-114118, 310020-135028; **Grant sponsors:** Cancer League of the Canton Zurich, "Forschungskredit" of the University of Zurich, "Stiftung Krebsforschung," "Stiftung für Wissenschaftliche Forschung and der Universität Zürich"
DOI: 10.1002/ijc.28239
History: Received 30 Nov 2012; Accepted 15 Apr 2013; Online 2 May 2013
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Int. J. Cancer: **133**, 2341–2350 (2013)

What's new?

Lymphoproliferative diseases associated with Epstein-Barr virus (EBV) infection cause morbidity and mortality in bone marrow and solid organ transplant recipients. Here the authors analyzed the early molecular events leading to B-cell transformation following EBV infection. They demonstrated in vitro, 72 hours post-infection when B-cell hyperproliferation starts, the up-regulation of cell cycle-related genes (CDKs) and of the apoptosis inhibitor survivin. Treatment with specific CDK or survivin inhibitors led to EBV-transformed B-cell death. Importantly, survivin over-expression was also found in EBV-positive post-transplant diffuse large B-cell lymphomas, suggesting that inhibition of CDKs or survivin could represent a potential therapy for EBV-associated lymphoproliferative diseases.

the leading malignancy in children after solid organ transplantation.[4] Primary EBV infection of the EBV-seronegative allograft recipient may be acquired *via* the palatine tonsils, the natural portal of entry for the virus,[5] or *via* EBV-infected donor-derived B-cells within the transplant.[6] Thus, inexistent or heavily impaired pre-existing EBV-specific immune control is an essential prerequisite for the pathogenesis of EBV-LPD that may emerge from recently EBV-infected B-cells derived from the natural portal of entry or from remote lymphatic organs.

In vitro, EBV unfolds a unique capacity to growth-transform B-cells, both in mononuclear cells derived from EBV-seronegative donors and in mononuclear cells from EBV-seropositive donors treated with immunosuppressants.[7] Thus, inexistent or impaired cellular EBV-specific immune control, as in EBV-LPD, results in unrestricted proliferation of B-cells newly infected with EBV that, by default, establishes a latent infection.[8] This suggests that similar pathogenic mechanisms may contribute to EBV-LPD *in vivo* and EBV-induced B-cell transformation *in vitro*.

Indeed, B-cells transformed by EBV *in vitro* have been instrumental in the study and characterization of the immune control of EBV, allowing demonstrating that control of latently EBV-infected B-cells by T-cells is essential. In this sense, B-cells transformed *in vitro* by EBV have been crucial for the engineering of EBV-specific cytotoxic T-cells (CTLs) to be used for adoptive transfer to patients to treat their EBV-LPD.[9] Notably, adoptive transfer of CTLs has proven successful in fighting established EBV-LPD. Nevertheless, the complexity of manufacturing EBV-specific CTLs restricts their availability to few specialized centers. Other, more widely used treatments of post-transplant EBV-LPD include lowering of drug-induced immunosuppression or transfusion of antibodies against B-cells.[10] Lowering immunosuppression, however, may not be applicable in BMT and is burdened with the risk of allograft rejection after SOT. Unspecific depletion of B-cells by anti-B-cell antibodies, on the other hand, may cause potentially deleterious B-cell deficiency. Also, prevention of EBV-LPD by, *e.g.*, avoiding certain compounds for conditioning BMT recipients or antibodies against T-cells may be rather problem-prone, although it may contribute to improve the patients' outcome.[1] Thus, although prevention and treatment of post-transplant EBV-LPD focus on either specific or unspecific control of EBV, its management remains challenging.

A novel approach to fight post-transplant EBV-LPD could be targeting molecular events leading to transformation that are imposed on B-cells by the virus early after infection. B-cells from post-transplant EBV-LPD lesions express the same pattern of EBV genes (including EBNAs, LMPs and EBERs) observed during *in vitro* B-cell transformation by EBV leading to the formation of lymphoblastoid cell lines (LCL).[7] Notably, the efficiency of EBV to growth-transform B-cells *in vitro* using the classic growth-transformation assay may differ considerably depending on the B-cell status of differentiation.[11] Thus, processes during EBV-induced B-cell transformation *in vitro* may mirror the situation in the immunosuppressed transplant patient and their analysis including B-cells from distinct tissues could lead to the identification of critical pathogenic steps that could be prophylactically or therapeutically targeted.

The aim of our work was to elucidate the early steps involved in EBV-induced B-cell transformation after infection with the virus. We used B-cells isolated from palatine tonsils from EBV-seronegative individuals. This enabled us to avoid using immune suppressants to counteract pre-existing EBV-specific CTLs and thus potential effects of immune suppressants on host gene expression. Furthermore, these samples enabled us to model the setting with the highest risk for the development of EBV-LPD, *i.e.*, primary EBV infection. We used microarray technology to profile host gene expression to identify genes involved in EBV-mediated B-cell transformation. Immunohistochemical analysis of post-transplant EBV-LPD confirmed the expression in patient samples and subsequent functional experiments allowed the identification of potential host targets to efficiently counteract EBV-LPD.

Methods

Ethics statement

Our study was conducted according to the principles expressed in the Declaration of Helsinki. Written informed consent for the collection of samples and subsequent analysis was obtained from next of kin, caretakers or guardians on behalf of the minors/children participants involved in our study. The Institutional Review Board of the University

Children's Hospital of Zurich approved the study (IRB study StV29/06).

Isolations of B-cells

Human mononuclear cells were isolated from tonsils of donors undergoing routine tonsillectomy as previously described.[12] B-cells were isolated using the B-cell isolation kit II according to the manufacturer's instructions (Miltenyi Biotech, Bergisch Gladbach, Germany). Purity of the B-cells was above 95% in all the preparations.

EBV infection

EBV infection was performed as described earlier[12] with minor modifications. Briefly, supernatants of B95.8 cells were centrifuged at 40,000*g* for 2 hr at 4°C. The pellet was resuspended in phosphate buffered saline and 10% of high-titer EBV was added to the cells.

EBER-1 *in situ* hybridization

The presence of EBER-1 in single cells was checked for by *in situ* hybridization and flow cytometry as reported,[13] but with the modification that the EBER-1 hybridization probe was linked to Atto620 (Microsynth, Balgach, Switzerland).

Microarrays

Two samples, from two independent donors, were used per each experiment. Infected and noninfected purified B-cells were collected after 72 hr. RNA was isolated from the cells at the indicated time point by phenol-chloroform extraction using Trizol followed by additional column purification using the RNeasy mini kit (Qiagene, Hombrechtikon, Switzerland). Integrity of RNA was determined by Bioanalyzer (Agilent, Basel, Switzerland) and transcribed to cRNA using the Ovation Pico WTA system (NuGEN, Leek, The Netherlands). Hybridization on Affymetrix HG-U133 Plus 2 chip was performed at the Functional Genomic Centre Zurich. Data quality control and extraction were performed in the B-Fabric platform, which uses several R scripts based on bioConductor.[14,15] RMA algorithm was used for summarization[16] and standard RMA.[17] Processed data were imported into the GeneGO portal for pathways enrichment analysis (MetaCore from GeneGo, Encinidas, CA). Microarray data were submitted to ArrayExpress (accession: E-MEXP-3582).

Cells treatments and analysis

Four LCLs were established from tonsillar mononuclear cells derived from four different EBV-negative donors, by infection with B95.8 EBV. Cells were kept in culture for 4–12 weeks. LCLs were split to a concentration of 0.3×10^6 cells/ml 16 hr before treatment; primary B-cells isolated from two donors were kept in culture 16 hr at 1.0×10^6 cells/ml in 100 µl volume. Increasing concentrations of flavopiridol, PD-0332991, YM155 (all from Selleck Chemicals, Houston, TX) and Terameprocol (Sigma-Aldrich, Buchs, Switzerland) dissolved 10 mM in dimethyl sulfoxide (DMSO) as a stock solution or corresponding maximum volume of DMSO as control were added to the cells. Cells were collected after 24 and/or 48 hr and subjected to measurement of proliferation, cell cycle analysis, detection of apoptosis and Western blotting as follows: measurement of proliferation was performed using an MTS assay (CellTiter 96 AQ_{ueous} One Solution Cell Proliferation Assay) according to the instructions of the manufacturer (Promega, Dubendorf, Switzerland). For cell cycle analysis, cells were harvested, washed in cold PBS and fixed in 70% ethanol. DNA was stained by incubating the cells in PBS containing propidium iodide (50 µg/ml, Sigma-Aldrich) and RNase A (100 µg/ml, Sigma-Aldrich) for 30 min at 37°C. Detection of apoptosis was performed using a PE Annexin V Apoptosis Detection Kit I according to the instructions of the manufacturer (Becton Dickinson, Allschwil, Switzerland). Data on cell cycle and apoptosis were collected using a fluorecence activated cell sorter FACSCanto II (Becton Dickinson) and analyzed using FlowJo software. To perform Western blotting, cell extracts were prepared in Laemmli buffer (4% Sodium dodecyl sulfate, 20% glycerol and 120 mM Tris, pH = 6.8). Electrophoresis and transfer to a membrane were performed using a NuPAGE system (Invitrogen). The antibodies used for immunoblots were rabbit anti-survivin (71G4B7, Cell Signaling Technology, Danvers, MA) and mouse anti-α-tubulin (DM1A, Sigma-Aldrich). For 5-(and-6)-carboxyfluorescein diacetate succinimidyl ester (CFSE) labeling, isolated B-cells were stained with 5 µM CFSE (Sigma-Aldrich) for 5 min at room temperature and then washed three times in PBS. CFSE-stained B-cells were infected with B95.8 EBV as already described and incubated at 37°C in 5% CO_2 air of relative humidity > 95% for indicated period. Then, the cells were subjected to flow cytometry using a FACSCanto II (Becton Dickinson) and the data were analyzed using FlowJo software.

Histology, preparation of tissue microarray and immunohistochemistry

Forty-eight biopsy specimens fixed in formalin and embedded in paraffin were collected and using a dedicated tool (microArrayer, Beecher Instruments, Sun Prairie, WI) tissue microarrays (TMAs) were constructed with 2 coring of 1-mm diameter from the blocks. From the obtained block new sections of 4 µm were cut (Supporting Information Fig. 6). Further details on patient samples and histological analysis can be found in Supporting Information Figs. 6 and 7 and Tables III and IV.

Statistical analysis

All statistical analysis was performed using Prism software (GraphPad Software) or Excel (Microsoft). Comparisons between groups were performed with an unpaired two-tailed Student's *t*-test. p Values < 0.05 were regarded as statistically significant, unless otherwise stated.

Results

Cellular gene expression in B-cells after exposure to EBV

To elucidate the very early steps involved in EBV-induced B-cell transformation after cellular infection with EBV, we set out to profile cellular gene expression in B-cells derived from

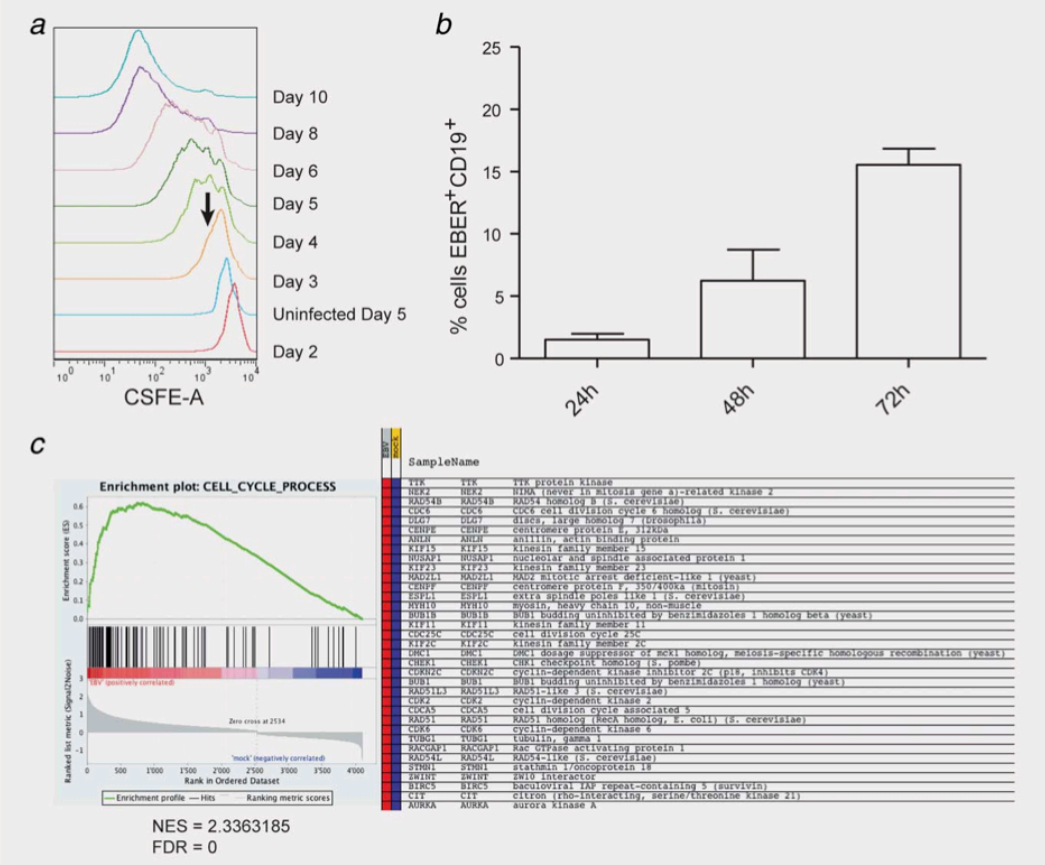

Figure 1. EBV infection efficiency of B-cells isolated from tonsils, proliferation analysis and gene set enrichment analysis of differentially regulated genes. (*a*) CSFE analysis of tonsillar B-cells (TBC). Cells were purified as described, loaded with CSFE, infected (or not) with EBV and at 24-hr intervals collected and analyzed by fluorescence activated cell sorting starting at day 2. The black arrow indicates the population of cells, which have already divided more than once. (*b*) Infection of B-cells by EBV was quantified by *in situ* flow cytometry for EBER-1 expression in tonsillar mononuclear cells infected with EBV monitored in CD19-positive cells at 24, 48 and 72 hr postinfection. For each time point the mean of two independent experiments conducted in triplicate is shown. (*c*) GSEA was performed on the set of genes differentially regulated in B-cells 72 hr after EBV infection. Twofold differentially regulated genes at $p < 0.01$ ($n = 1213$) were included in the GSEA and analyzed against the Gene Ontology sets (c5.all.v3.1.symbols). Forty-seven gene sets were upregulated and eight were downregulated at a false discovery rate (FDR) <25%. Left, shown is the gene set with the highest NES (NES = 2.3). Right, the list of the top regulated genes within the cell cycle process gene set.

tonsils of EBV-seronegative individuals and newly infected with EBV *ex vivo*. Tonsils are considered as the portal of entry of EBV in the body. We chose 72 hr after infection as time point of analysis, because at this time infected B-cells start undergoing a phase of hyperproliferation lasting up to 3 days (Fig. 1*a*) that has been correlated with a phase of heightened oncogenic activity leading to B-cell transformation.[18] Knowing the proportion of EBV-infected B-cells at the time of analysis is important to judge the accuracy of gene expression analysis. By measuring EBER-1 expression, a surrogate marker for EBV infection, by flow cytometry we detected around 15% EBV positive B-cells after 72 hr (Fig. 1*b* and Supporting Information Fig. 1). Thus, 3 days postexposure to EBV *in vitro*, up to one-fifth of the B-cells harbored EBV and would undergo hyperproliferation. Noninfected autologous tonsillar B-cells cultured for 72 hr were taken as baseline, and B-cell gene expression was assessed on an Affymetrix Chip HG-U133 Plus 2 (EBV-infected samples $n = 2$; mock-infected samples $n = 2$).

At stringent selection conditions ($p < 0.001$) we detected 143 twofold differentially regulated genes, 1,213 genes with $p < 0.01$, and at $p < 0.05$ 2,482 genes were found to be

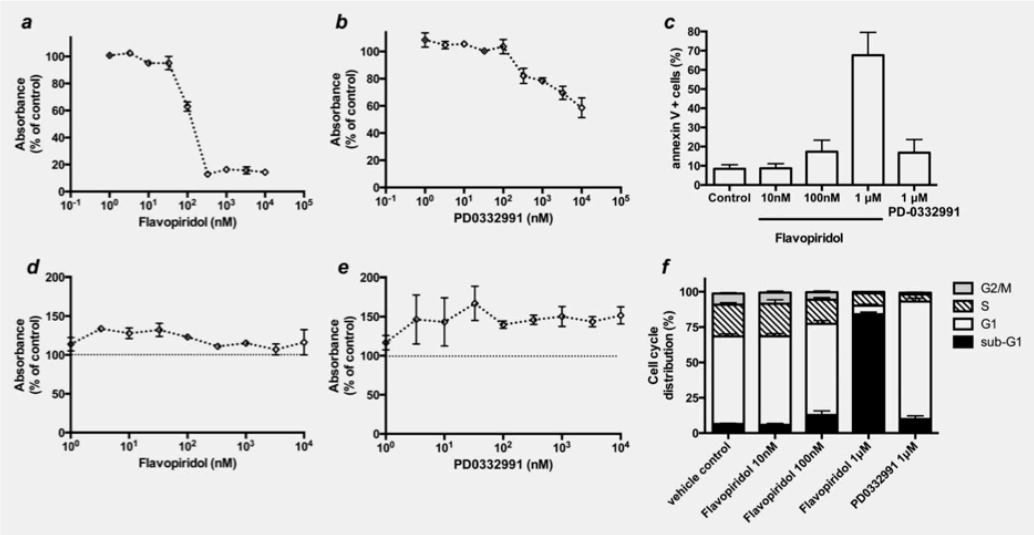

Figure 2. Treatment of EBV-transformed B-cells with CDK inhibitors reduces cell viability. (*a*) Treatment of four independent LCLs with increasing concentrations of flavopiridol for 48 hr resulted in a dose-dependent decrease in cell proliferation with IC_{50} ranging from 100 to 330 nM. (*b*) On the other hand, treatment of LCLs with PD-0332991 for 48 hr also resulted in a dose-dependent decrease in cell proliferation but weaker than with flavopiridol. (*c*) Analysis of Annexin V positivity by FACS showed a strong increase in cells treated with 1 μM flavopiridol after 48 hr. (*d*) The effect of flavopiridol on EBV-negative cells was tested on tonsillar B-cells purified from two donors incubated with increasing concentrations of flavopiridol for 48 hr. Cell proliferation was measured and compared to vehicle-treated cells. (*e*) Similarly, the effect of increasing PD0332991 on EBV-negative cells proliferation was tested. (*f*) Analysis of cell cycle distribution confirmed a significant increase of apoptotic cell fraction (sub-G1) after treatment with 1 μM flavopiridol and suggested a strong G1 accumulation for LCLs treated with 1 μM PD0332991 after 48 hr. The results shown are from one of two experiments performed with four LCLs in duplicate.

differentially regulated more than twofold by EBV infection in tonsillar B-cells. To identify important EBV-associated early cellular molecular changes leading to transformation of B-cells we performed gene set enrichment analysis (GSEA) on the differentially regulated genes at $p < 0.01$. A striking enrichment of genes related to cell cycle was revealed (Fig. 1*c*, right panel). Analysis of the differentially regulated genes using the GeneGO Pathway maps confirmed the impact of EBV infection on the expression of cell cycle-related genes, but also pointed out to a significant impact on apoptosis (Supporting Information Fig. 2 and Table 1). Interestingly, among the highest ranked genes in the "Cell Cycle Phase" GSEA we found BIRC5 (survivin), a regulator of cell cycle and strong antiapoptotic protein (Fig. 1*c*, left panel and Supporting Information Table 2). Thus, the identity of genes differentially regulated by EBV infection in B-cells at the time of earliest signs of transformation indicated a significant impact on cell cycle and apoptosis.

Cyclin-dependent kinases essentially contribute to EBV-mediated B-cells transformation *in vitro*

We observed a clear and robust upregulation of genes associated with cell cycle or cell proliferation in B-cells after infection with EBV. Strikingly, cyclin-dependent kinases (CDKs) 1, 2, 4 and 6 were consistently upregulated in B-cells derived from tonsils 72 hr postinfection with EBV (Table 1 and Supporting Information Fig. 2). Upregulated expression of CDK1, CDK2 and CCNB1 was confirmed by real time quantitative polymerase chain reaction (qPCR) (Supporting Information Fig. 5). Expression of CDKs is also higher in transformed LCLs, *i.e.*, >30 days after B-cells infection with EBV, compared to primary B-cells as judged by microarray profiling, however, to a lesser extent than at 72 hr after infection (data not shown).

Table 1. Expression changes of selected cell cycle-related genes in tonsillar B-cells upon EBV infection *ex vivo*

Gene symbol	Expression change	*p*-value
CDK1	4.76	0.03
CDK2	2.85	0.01
CDK4	1.84	0.01
CDK5R1	3.32	0.00
CDK5RAP1	0.75	0.01
CDK6	3.40	0.05
CDK8	2.30	0.01
CCNB1	3.18	0.04

Expression change values depicted are $\log_2$ ratios of EBV-infected *vs.* uninfected cells at 72 hr.

Table 2. Top ten differentially regulated apoptosis-related genes in tonsillar B-cells upon EBV infection *ex vivo*

Gene symbols	Expression change	*p*-value
BIRC5	4.11	0.04
EPR1	3.82	0.01
BRCA1	3.71	0.02
BID	2.69	0.02
ZAK	2.39	0.02
STAT1	2.37	0.00
CYCS	2.19	0.01
TNFRSF21	2.15	0.05
DHCR24	2.10	0.00
CHEK2	2.04	0.01
PRKCE	−1.38	0.01
FOXO3	−1.41	0.05
HRK	−1.67	0.04
MAPK1	−1.75	0.04
BNIP3L	−1.78	0.02
FOXO1	−1.98	0.01
CASP8	−2.24	0.05
CD40	−2.25	0.05
PSEN1	−2.49	0.01
HSPA1A	−3.00	0.01

Expression change values depicted are $\log_2$ ratios of EBV-infected *vs.* uninfected cells at 72 hr.

CDKs represent an appealing target for therapeutic intervention to eliminate EBV-mediated expansion of EBV-infected or EBV-transformed B-cells through a decrease of proliferation. Thus, to test the effect of specific CDK inhibitors on EBV-transformed B-cells, we treated four LCLs established from *in vitro* EBV-infected tonsillar B-cells, kept in culture for at least 30 days, with increasing concentrations of flavopiridol, a pan-CDK inhibitor, and PD0332991, a specific CDK4 and CDK6 inhibitor; both compounds are currently in phase II clinical trials.[19,20] Cell viability assessed by MTS assay 48 hr after treatment indicated that all LCLs were sensitive to flavopiridol because viability dropped to 20% of control (Fig. 2*a*). PD0332991, however, was not as effective in diminishing LCL viability reaching only 80% of control at the highest concentration of 1 μM (Fig. 2*b*). To verify whether the reduction in cell viability was owing to activation of apoptosis, we analyzed Annexin V exposure after 48 hr treatment with 1 μM flavopiridol. Indeed, more than 60% of the cells stained positive for Annexin V, indicating an activation of apoptotic response (Fig. 2*c*). Survival of EBV-negative B-cells was not affected by flavopiridol (Fig. 2*d*) or by PD0332991 (Fig. 2*e*) even at the higher concentrations. Cell cycle distribution analysis of LCLs treated with increasing concentrations of flavopiridol showed a large increase of the sub-G1 population upon treatment with 1 μM flavopiridol at 48 hr (Fig. 2*f*), confirming that apoptosis might be the main mechanism responsible for flavopiridol-induced reduction of cell viability. Further, cell cycle analysis of LCLs treated with 1 μM PD0332991 showed an accumulation in G1 and decrease in S and G2 phases suggesting G1 arrest (Fig. 2*f*) with a rather modest impact on cell survival. Taken together, these results indicated that flavopiridol is more effective than PD0332991 in reducing viability of LCLs, and suggested that targeting CDKs, particularly CDK1, might be an effective approach to control and eliminate EBV-transformed B-cells.

EBV infection increases expression of antiapoptotic genes in tonsillar B-cells

EBV is known to inhibit apoptosis during latent infection.[21] Thus, we aimed to identify additional apoptosis-related genes that are affected during the early events of EBV-mediated B-cell transformation. Indeed, BIRC5, coding for the strong antiapoptotic protein survivin,[22] showed the highest upregulation (17-fold) among the ten most highly upregulated apoptosis-related genes (Table 2). BIRC5 (survivin) is a target of CDK1 and cyclin B1 (CCNB1) phosphorylation,[23,24] both of which are also upregulated upon EBV infection (Table 1 and Supporting Information Fig. 3). We confirmed 4.5-fold upregulation of BIRC5 expression by EBV infection in tonsillar B-cells using real-time qPCR (Supporting Information Fig. 5). Taken together, these results suggest that CDK1-CCNB1 and survivin represent an important signaling axis for the survival of EBV-infected B-cells.

Survivin is rather consistently expressed in EBV-positive post-transplant EBV-LPD

To investigate whether survivin is also expressed in EBV-positive B-cell lymphomas *in vivo*, we generated TMA from a panel of 30 post-transplant EBV-LPD (Supporting Information Figs. 6 and 7 and Table 3). Presence of EBV was ascertained by EBER *in situ* hybridization (Fig. 3*a*) and by LMP1 staining (Supporting Information Fig. 7n). Expression of survivin was assessed by IHC, and detection in more than 30% of the EBV-LPD cells was defined as positive (Fig. 3*b*). Seventy-one percent ($n = 17$) of the evaluable EBV-positive tumors expressed survivin (Supporting Information Table 4). These results documented that survivin is consistently upregulated in EBV-infected cells and indicated that survivin may have and important impact on B-cells transformation and proliferation *in vivo* when the immune system is compromised.

Survivin inhibition decreases survival of EBV-transformed B-cells

To verify whether survivin expression is important for survival and proliferation of *in vitro* EBV-transformed B-cells, we tested the effect of two inhibitors of survivin, YM155 and terameprocol, on four freshly established LCLs. Both compounds act at the transcription level by blocking Sp1 activity on the survivin promoter.[25] Cells were incubated with

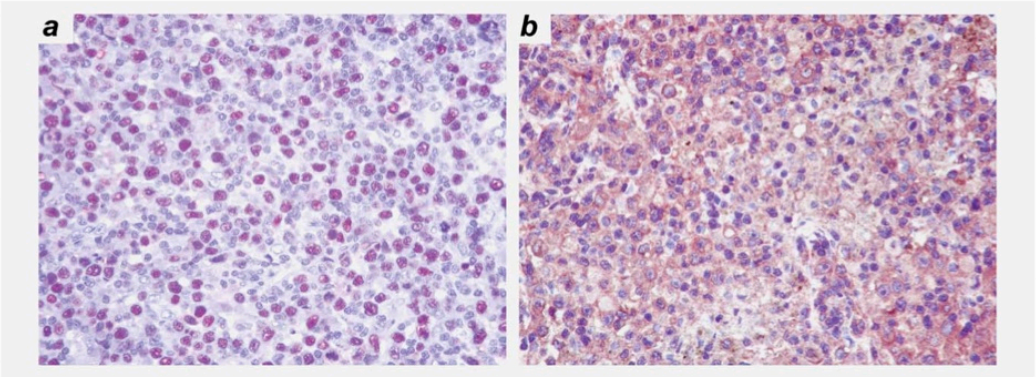

Figure 3. Expression of survivin in EBV-positive EBV-LPD. (*a*) Representative result for positive EBER *in situ* hybridization with DNA ISH Detection (Dako, Golstrup, Denmark) and (*b*) for positive staining for survivin expression developed with Envision + (Dako). Samples were considered to be positive for survivin when more than 30% of the neoplastic cells stained positive. Magnification, ×400.

increasing concentrations of YM155 or terameprocol and cell proliferation was monitored after 48 hr by MTS assay (Fig. 4). Treatment with increasing concentrations of YM155 resulted in a significant decrease of LCL viability (Fig. 4*a*). One nanomolar YM155 reduced cell viability by 20% and 10 nM YM155 reduced cell viability to less than 20%. At the highest concentration tested (1 μM YM155) all cells were dead. The estimated IC_{50} for YM155 was 2.6 nM. Likewise, the treatment with increasing concentrations of terameprocol resulted in decreased cell viability, with an estimated IC_{50} of 46.4 μM (Fig. 4*b*), in good agreement with other reports.[26] Analysis of preapoptotic cells exposing Annexin V confirmed induction of apoptosis in 30% of the cells by treatment with 10 nM YM155 and up to 70% with 100 nM YM155 or 100 μM terameprocol (Fig. 4*c*). It is noteworthy that treatment with 10 nM YM155 resulted in 80% decrease of cell viability, but only 30% of the cells were positive for Annexin V, suggesting a differential impact of YM155 on proliferation and apoptosis. Survival of EBV-negative B-cells was not affected by treatment with YM155 (Fig. 4*d*) or terameprocol (Fig. 4*e*). Only at the highest YM155 concentration of 1 μM cell viability was reduced to 80%, but at the YM155 IC_{50} concentration of 2.6 nM the viability of EBV-negative B-cells was not affected. Successful suppression of survivin expression was verified at the mRNA level by qPCR (Supporting Information Fig. 8) and at the protein level by Western blotting (Fig. 4*f*). A significant decrease of mRNA expression was observed after 24-hr treatment with 10 or 100 nM YM155. Survivin protein decrease was evident by Western blotting after 24-hr treatment with 100 μM terameprocol and after 48-hr treatment with 10 and 100 nM YM155 (Fig. 4*f*). These results indicated that YM155 is more efficient than terameprocol in decreasing survivin levels, and that treatment of EBV-transformed B-cells with survivin inhibitors results in induction of apoptosis and consistent decrease in cell survival.

Discussion

LPD associated with the B-cell tropic EBV causes significant morbidity and mortality in BMT[2] or SOT recipients.[1] An improved understanding of the early molecular events after EBV infection leading to B-cell transformation is essential to gain insight into LPD pathogenesis and help devise new therapeutic approaches. Here, we have analyzed the impact of EBV infection on early host gene expression in B-cells purified from tonsils, the portal of entry for EBV in the body. We investigated B-cells from EBV-negative individuals to mimic molecular events occurring during primary EBV infection of EBV-negative transplant recipients, the subpopulation with the highest risk for post-transplant EBV-LPD. Among the upregulated genes we identified in particular CDKs (CDK1/2/4/6), CCNB1 and the CDK1/CCNB1 target BIRC5 (survivin), an inhibitor of apoptosis, which we found highly expressed in established LCLs and in the majority of EBV-LPD tumors. Given that specific survivin inhibitors efficiently counteracted proliferation of EBV-transformed B-cells, CDK and survivin represent potential novel therapeutic targets for EBV-LPD.

Cellular transformation induced by EBV infection has been subject of active investigation by many groups. The mechanisms underlying cellular transformation by EBV have been investigated either by using wild-type virus,[18,27–30] recombinant virus mutated in one or more genes[31–33] or by ectopic expression of single EBV proteins known to be expressed in EBV-transformed cells (*e.g.*, EBNA1[34,35] and EBNA2[36–39]). Gene expression profiling of infected primary B-cells or of modified B-cell lines has revealed a number of genes whose expression is directly or indirectly influenced by

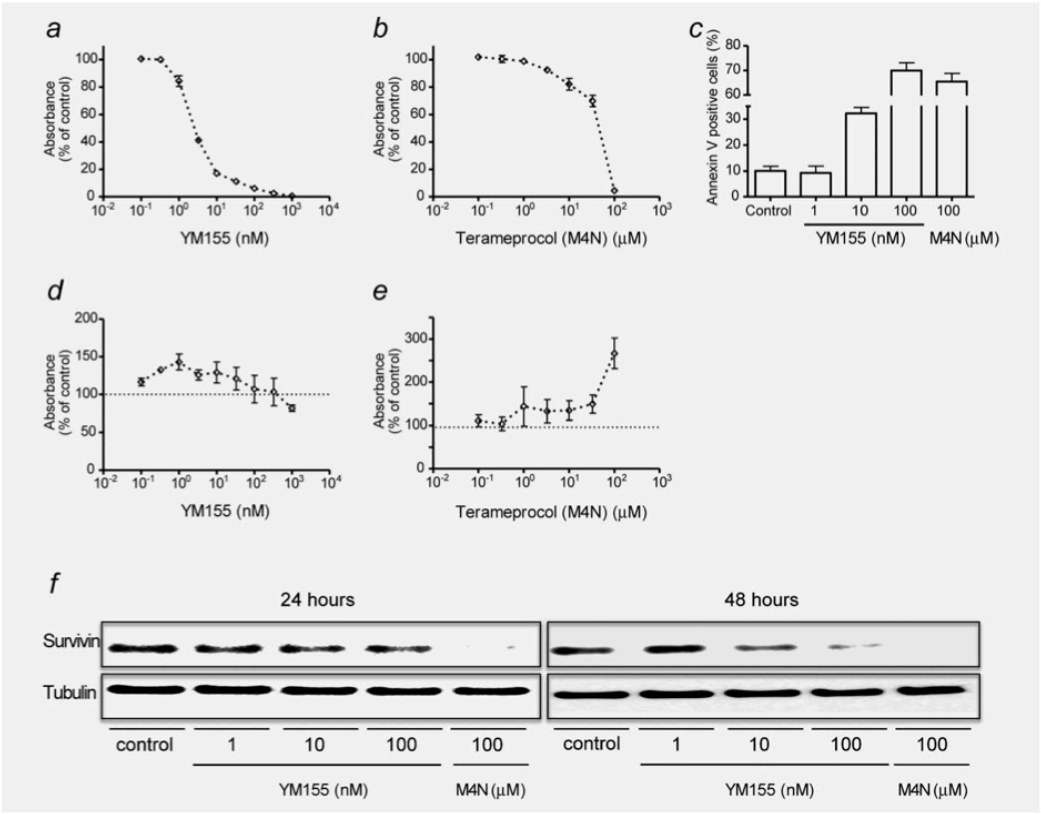

Figure 4. Survivin inhibition by YM155 or terameprocol decreases survival of EBV-transformed B-cells through apoptosis. Four independent LCLs were treated with increasing concentrations of survivin inhibitors YM155 and terameprocol (M4N) and survival was measured by MTS assay 48 hr after treatment. (*a*) Cell proliferation normalized to untreated control cells after incubation with 0.1, 0.33, 1.0, 3.3, 10, 33, 100, 333 and 1,000 nM of YM155 and (*b*) with 0.1, 0.33, 1.0, 3.3, 10, 33, 100, 333 and 1,000 μM of terameprocol. (*c*) Quantification of apoptotic cells by FACS analysis of Annexin V-positive cells after 48 hr of treatment. (*d*) The effect of YM155 on EBV-negative cells was tested on tonsillar B-cells purified from two donors incubated with increasing concentrations of YM155 for 48 hr. Cell proliferation was measured and compared to vehicle-treated cells. (*e*) Similarly, the effect of increasing terameprocol on EBV-negative cell proliferation was tested. (*f*) Effective inhibition of survivin expression was verified by Western blot of cells incubated with 1–100 nM YM155 or 100 μM terameprocol (M4N). Tubulin was used as loading control. The results shown are from one of two experiments performed with four LCLs in duplicate.

EBV proteins. It is not surprising that by using different profiling methods not always overlapping results have been obtained. In addition, differential activity in different cellular backgrounds has been shown, *e.g.*, for EBNA1.[40] However, some patterns seem to emerge as to the impact of EBV infection on cellular gene expression, which are congruent with our results. EBNA2 is the first EBV gene to be detected after infection and at 72 hr postinfection we previously measured expression of most latency-associated EBV genes,[12] in good agreement with that we found differential expression of validated EBNA2 targets such as CDK2, 4 and 6,[41] and EBNA1 target genes such as BIRC5.[34] The fact that we find no EBNA3 or LMP1 target genes suggests that the impact of these proteins on cellular gene expression is less pronounced at 72 hr after infection. Indeed, LMP1 activation of NFκβ signaling is surprisingly delayed during primary EBV infection of B-cells.[30]

Our results, indicating an important role of cell cycle regulators early during EBV infection and transformation, in particular of CDKs 1, 2, 4 and 6, urged us to test the impact of their inhibition on LCLs. Of the two inhibitors tested, flavopiridol, a pan-CDK inhibitor, was more effective than PD0332991, specific for CDK4 and CDK6, suggesting that CDK1 or CDK2 activity is more important for survival of EBV-transformed B-cells and might be more effective than CDK4 or CDK6 in EBV-LPDs. This is in good agreement

with the decreased expression of CDK4 and CDK6 and the sustained overexpression of CDK1 and CDK2 LCLs observed 30 days after infection (M. Bernasconi, unpublished observation). LCLs sensitivity to flavopiridol might also be explained by inhibition of CDK9-cyclin T1 (P-TEFb)[42] and subsequent decreased activation of EBV promoter Cp and EBV latent gene EBNA1 and EBNA2 transcription, as shown in Burkitt's lymphoma cells,[43] might lead to decreased survival and offer a second layer of specificity in EBV-PTLD.

The importance of CDK1 in EBV-driven B-cell transformation is also supported by the identification of survivin, a CDK1-cyclin B target, as an important antiapoptotic factor upregulated early upon EBV infection. The complex of CDK1-cyclin B phosphorylates survivin at Thr34. In the absence of Thr34 phosphorylation survivin is rapidly degraded resulting in disruption of survivin-caspase 9 complexes leading to increased caspase 9-dependent apoptosis in the M phase.[23,24] Thus, in addition to CDK inhibitors, inhibitors of survivin expression could also represent important therapeutic compounds for the treatment of early and more advanced stages of EBV-LPD. Importantly, survivin expression is increased in LCLs when compared to EBV-negative B-cells. Indeed, inhibition of survivin expression using YM155 or terameprocol was effective in killing LCLs, a model for post-transplant EBV-LPD, suggesting an important involvement of survivin in EBV-driven B-cell transformation. Interestingly, lower concentrations of YM155 (10 nM) significantly reduced LCLs proliferation without inducing apoptosis, whereas higher concentrations (>100 nM) strongly induced apoptosis suggesting a dual function, proliferative and antiapoptotic, of survivin in LCLs. Notably, survivin plays a significant role in other EBV-associated malignancies such as gastric carcinoma[44] and NK/T cell lymphoma.[26] Increased expression of survivin upon EBV infection in B-cells and in post-transplant EBV-LPD is novel. The collaboration of latent EBV protein EBNA1 with Sp1 to increase survivin expression by binding to the survivin promoter might provide a mechanistic explanation for upregulation of survivin in EBV-positive tumors.[34] The importance of survivin in PTLPD is further highlighted by the fact that also EBV-negative DLBCL express survivin, as shown here and by others,[45] indicating that additional molecular mechanisms might be responsible for increased survivin expression in EBV-PTLD, usually arising within 1 year of transplant, and other PTLD arising later after organ transplantation. In addition, given the importance of EBV infection in pediatric diffuse large B-cell lymphomas,[46] it is imperative that therapies designed to target cell cycle and survivin are considered for EBV-positive PTLDs, which are often resistant to the current therapies that consist in a combination of chemotherapy (CHOP) and rituximab, a chimeric anti-CD20 monoclonal antibody.[47]

Collectively, in view of the suppressive effect of CDK and survivin inhibitors *in vitro* on proliferation and survival of LCLs documented here, it is tempting to propose studies in high-risk patients for LPD like EBV-seronegative transplant recipients. Notably, YM155 has shown a good pharmacological profile in clinical trials,[48] and flavopiridol is currently in phase II clinical trial for relapsed mantle cell lymphoma and DLBCL (NCT00445341). Given that lowering of immunosuppression is not possible in BMT recipients and administration of radio/chemotherapy with or without anti-B-cell antibodies puts the transplant at risk, our findings provide the rationale for additional studies on the role of EBV on survivin and CDKs regulation and malignant cells survival in PTLD that might lead to tailored interventions based on specific survivin and/or CDK inhibitors such as YM155 and flavopiridol.

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Lentiviral Transduction Particles:

Lentiviral transduction particles from Sigma Aldrich

Particles	Order number	Sequences	Name	TU/ml
Control particles	SHC002V MISSION		Non-target shRNA	$2.6x10^7$
	SHC003V MISSION		TurboGFP control particles	$2.7x10^7$
	SHC008V MISSION		B2M (beta 2 microtubulin)	$3.2x10^7$
SHCLNV-NM_00085	TRCN0000029654	S1	α4	$3.7x10^6$
	TRCN0000029655	S2		$4.5x10^6$
	TRCN0000029656	S3		$5.4x10^6$
	TRCN0000029657	S4		$5.6x10^6$
	TRCN0000029658	S5		$9.9x10^6$
SHCLNV-NM_001006658	TRCN0000057113	S1	CD21	$8.9x10^6$
	TRCN0000057114	S2		$2.6x10^6$
	TRCN0000057115	S3		$6.5x10^6$
	TRCN0000057116	S4		$3.3x10^6$
	TRCN0000057117	S5		$4.1x10^6$